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Phone: 571-272-2524

maryjane.ruhl@uspto.gov

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From: Parkin, Jeffrey
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Please search **SEQ ID NOS.: 1 and 2** (U.S. Serial No. **10/085,944**) from the aforementioned application v. all relevant databases. Place results on both paper and disk. Thanks.

JSP
AU 1648
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CRF-E

REPLACEMENT
SEARCH

Amendments to the Claims:

This listing of claims replaces all prior versions and listings of claims in the application:

Listing of Claims:

1-14. (Withdrawn)

INDICATE CLOSED IN NOA

O.K. 15. (Original) A kit for detecting dengue virus comprising:

A first dengue virus-specific primer, which is 18 to 28 nucleotides in length and includes at least 18 nucleotides of SEQ ID NO:1; and

A second dengue virus-specific primer, which is 18 to 28 nucleotides in length and includes at least 18 nucleotides of SEQ ID NO:2.

O.K. 16. (Original) The kit of claim 15, further comprising a known amount of a competitor nucleic acid with length detectably different from the dengue virus RNA.

O.K. 17. (Original) The kit of claim 15, wherein the first dengue virus-specific primer is 18 to 23 nucleotides in length.

O.K. 18. (Original) The kit of claim 15, wherein the first dengue virus-specific primer is the nucleotide sequence of SEQ ID NO:1.

O.K. 19. (Original) The kit of claim 15, wherein the second dengue virus-specific primer is 18 to 23 nucleotides in length.

O.K. 20. (Original) The kit of claim 15, wherein the second dengue virus-specific primer is the nucleotide sequence of SEQ ID NO:2.

O.K. 21. (Original) The kit of claim 17, wherein the second dengue virus-specific primer is 18 to 23 nucleotides in length.

O.K. 22. (Original) The kit of claim 18, wherein the second dengue virus-specific primer is the nucleotide sequence of SEQ ID NO:2.

O.K. 23. (Original) A nucleic acid, which is 18 to 28 nucleotides in length and includes at least 18 consecutive nucleotides of SEQ ID NO:1.

O.K. 24. (Original) The nucleic acid of claim 23, wherein the nucleic acid is 18 to 23 nucleotides in length and includes at least 18 consecutive nucleotides of SEQ ID NO:1.

O.K. 25. (Original) The nucleic acid of claim 23, wherein the nucleic acid is the nucleotide sequence of SEQ ID NO:1.

NEW MATTER 26. (Currently Amended) A nucleic acid, which is 18 to ~~28~~ 25 nucleotides in length and includes at least 18 consecutive nucleotides of SEQ ID NO:2.

O.K. IF [illegible] 27. (Original) The nucleic acid of claim 26, wherein the nucleic acid is 18 to 23 nucleotides in length and includes at least 18 consecutive nucleotides of SEQ ID NO:2.

'276 ART 28. (Currently Amended) A ~~The~~ nucleic acid ~~of claim 26~~, wherein the nucleic acid is the nucleotide sequence of SEQ ID NO:2.

AMPLIFIED

29. (Original) An isolated nucleic acid comprising a fragment of a dengue viral genome or a DNA copy thereof, wherein the fragment includes:

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a first sequence that is complementary or identical to at least 18 consecutive nucleotides of SEQ ID NO:1;

a second sequence that is complementary or identical to at least 18 consecutive nucleotides of SEQ ID NO:2; and

a non-naturally occurring deletion or insertion, the deletion or insertion occurring in a region of the fragment flanked by the first and the second sequence.

30. (Original) The nucleic acid of claim 29, wherein the first sequence is complementary or identical to SEQ ID NO:1 and the second sequence that is complementary or identical to SEQ ID NO:2.

31. (New) The nucleic acid of claim 29, wherein the nucleic acid is consists of:

a first sequence that is complementary or identical to at least 18 consecutive nucleotides of SEQ ID NO:1;

a second sequence that is complementary or identical to at least 18 consecutive nucleotides of SEQ ID NO:2; and

a third sequence that is flanked by the first and second sequence, wherein the third sequence is complementary or identical to a genomic Dengue virus region that is (a) naturally flanked by the first and the second sequence and (b) includes a non-naturally occurring deletion or insertion.

32. (New) The nucleic acid of claim 31, wherein the first sequence is complementary or identical to SEQ ID NO:1 and the second sequence is complementary or identical to SEQ ID NO:2.

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(54) COMPOSITIONS AND METHODS FOR TREATING HEMORRHAGIC VIRUS INFECTIONS AND OTHER DISORDERS

(76) Inventors: Terry M. Fredeking, Bedford, TX (US); George M. Ignatyev, Koltsovo (RU)

Correspondence Address:
HELLER EHRMAN WHITE & MCAULIFFE LLP
4250 EXECUTIVE SQ
7TH FLOOR
LA JOLLA, CA 92037 (US)

(21) Appl. No.: 09/840,707

(22) Filed: Apr. 23, 2001

Related U.S. Application Data

(60) Division of application No. 09/562,979, filed on Apr. 27, 2000, which is a non-provisional of provisional application No. 60/198,210, filed on Apr. 27, 1999, now abandoned.

Publication Classification

(51) Int. Cl.^{7} A01N 37/18; A61K 38/00
(52) U.S. Cl. 514/2

(57) ABSTRACT

Cytokine-receptor and cytokine antagonist-enriched blood-dervided compositions and methods of preparing and using the compositions are provided. Also provided are compositions and methods for the treatment or prevention of disorders, especially acute inflammatory disorders involving pathological responses of the immune system, such as viral hemorrhagic diseases, sepsis, rheumatoid arthritis and other autoimmune disorders, acute cardiovascular events, flare-ups and acute phases of multiple sclerosis, wasting disorders and other disorders involving deleterious expression of cytokines and other factors, including tumor necrosis factor (TNF) and interleukin-1 (IL-1) are provided.

SEQ 23 (2 cont)

COMPOSITIONS AND METHODS FOR TREATING HEMORRHAGIC VIRUS INFECTIONS AND OTHER DISORDERS

RELATED APPLICATIONS

[0001] This application is a divisional of U.S. application Ser. No. 09/562,979, filed Apr. 27, 2000, by Terry M. Fredeking and George M. Ignatyev, entitled "COMPOSITIONS AND METHODS FOR TREATING HEMORRHAGIC VIRUS INFECTIONS AND OTHER DISORDERS". Benefit of priority under 35 U.S.C. §119(e) to U.S. provisional application Ser. No. 60/198,210, which was filed as U.S. application Ser. No. 09/301,274, filed Apr. 27, 1999, and converted to a provisional on Apr. 27, 2000, by Terry M. Fredeking and George M. Ignatyev, entitled "COMPOSITIONS AND METHODS FOR TREATING HEMORRHAGIC VIRUS INFECTIONS AND OTHER DISORDERS", is claimed herein.

[0002] The subject matter of each of U.S. application Ser. Nos. 09/562,979 and 09/301,274 is incorporated by reference in its entirety.

FIELD OF INVENTION

[0003] The present invention relates to compositions and methods for treating and/or preventing in mammals, particularly humans, acute inflammatory responses and diseases. More particularly, compositions and combinations of compositions and methods for the treatment of disorders, especially acute inflammatory disorders, involving pathological responses of the immune system are provided. Hence the disclosure herein provides compositions and methods for preventing and/or treating diseases, disorders and conditions that include viral hemorrhagic diseases and other acute infectious diseases, sepsis, rheumatoid arthritis and other autoimmune disorders, acute cardiovascular events, flare-ups and acute phases of multiple sclerosis, wasting disorders and other disorders involving deleterious expression of cytokines and other factors, including tumor necrosis factor (TNF) and interleukin-1 (IL-1).

BACKGROUND OF THE INVENTION

Diseases and Disorders Associated with or Characterized by Acute Inflammatory Responses

[0004] Responses of the immune system to pathogens and to other bodily insults are essential for survival of mammals. Inappropriate or excessive response, however, is associated with certain acute and chronic diseases. In such cases, inappropriate stimulation of various defense strategies involving inflammatory cells and the immune system produces the symptoms characteristic of the disease. The response of a mammal to infection with a hemorrhagic virus or a pathogenic strain of *Escherichia coli* and sepsis are exemplary of such responses. There are few, if any, effective treatments to counteract these responses.

Interleukin-1 and Receptors Therefor

[0005] The two forms of Interleukin-1 (IL-1α and IL-1β) are cytokines produced primarily by mononuclear phagocytes, but also by a number of other cell types including skin keratinocytes, some epithelial cells, and some cells of the central nervous system (CNS). These cytokines produce a wide variety of effects on numerous cell types, including the induction or suppression of the production of a great number of other proteins including interleukins, cytokines, tumor necrosis factors, and colony stimulating factors. IL-1α and IL-1β are thus important mediators of the inflammatory and immune responses of animals. Because of the early appearance of IL-1 during the inflammatory reaction and the immune response, and because of the variety of effects produced by IL-1α and IL-1β, these factors play a role in the production of pathological conditions resulting in chronic inflammation, septic shock, and defects in hematopoiesis. The effects of these interleukins result from the binding of these factors to two distinct cell surface receptors, IL-1R Types 1 and II. Type I receptor is an 80 kDa protein found on T cells, fibroblasts, and keratinocytes. Type II receptor is a 68 kDa protein found on B cells and polymorphonuclear leukocytes (PMNs). In general, the Type I receptor binds to IL-1α or IL-1β with approximately equal affinity and the Type II receptor binds IL-1β more strongly than IL-1α. Results indicate that only the Type I receptor is capable of transducing a signal and can produce all of the biological effects attributed to IL-1. It has been suggested that the function of the membrane-bound Type II receptor is to serve as the precursor for a soluble IL-1 binding factor that can be shed under appropriate circumstances to antagonize and modulate IL-1 activity. A naturally occurring IL-1 binding protein has been described that seems to correspond to the soluble external portion of the Type II receptor.

[0006] A different type of naturally occurring inhibitor of IL-1 activity was discovered and purified from the urine of patients with monocytic leukemia. A cDNA clone encoding this polypeptide has been isolated from monocytes and found to code for a mature 152 amino acid residue glycoprotein of 25,000 molecular weight. This molecule, known as secreted IL-1 receptor antagonist (sIL-1 Ra), shows 25% amino acid homology to IL-1β and 19% homology to IL-1α. Evidence indicates that the inhibitory action of sIL-1Ra results from binding of IL-1Ra to the IL-1 receptor Type I with an affinity comparable to that of IL-1α or IL-1β (Kd -200 pM), thus competing with IL-1α or β for binding to this receptor. This binding, however, does not result in signal transduction. IL-1Ra binds to the IL-1 receptor Type II with considerably lower affinity than that shown by IL-1β.

[0007] Cells know to produce IL-1ra include monocytes, neutrophils, macrophages and fibroblasts. Cytokines known to upregulate IL-1Ra production include IL-13, IL-6, IL-4, IFN-γ, GM-CSF and TGF-β, the latter apparently by triggering IL-1 production which itself triggers IL-1ra synthesis. The amino acid sequences of IL-1ra from at least four species have been determined (human, rat, mouse and rabbit) and found to be at least 75% homologous (Cominelli etal. (1994) *J. Biol. Chem.* 269:6963), IL-1ra can also be synthesized as a strictly intracellular form whose production is the result of an alternative splicing of exon 1 (Butcher et al. (1994) *J. Immunol.* 153:701; Arend et al. (1993) *Adv. Immunol.* 54:167). IL-1Ra is released in vivo during experimentally-induced inflammation and as part of the natural course of many diseases. Administered experimentally, IL-1Ra has been demonstrated to block IL-1 activity in vitro and in vivo.

Tumor Necrosis Factors and Receptors Therefor

[0008] Tumor necrosis factors (TNFs) are pleiotropic cytokines that are primary modifiers of the inflammatory and

immune reactions of animals produced in response to injury or infection. Two forms of TNF, designated TNF-α (or cachectin) and TNF-β (or lymphotoxin), have been described. These forms share 30% sequence similarity and compete for binding to the same receptors. TNFs play a necessary and beneficial role as mediators of host resistance to infections and tumor formation. Over production or inappropriate expression of these factors can lead to a variety of pathological conditions, including wasting, systemic toxicity, and septic shock (see, Beutler et al. (1988) *Ann. Rev. Biochem.* 57:505; and Vilcek et al. (1991) *J. Biol. Chem.* 266:7313).

[0009] The actions of TNFs are produced subsequent to binding of the factors to cell surface receptors. Two distinct TNF receptors have been identified and cloned. Virtually all cell types studied show the presence of one or both of these receptor types. One receptor type, termed TNFR-II (Type A, Type α, 75 kDa or utr antigen), has an apparent molecular weight of 75 kDa. The gene for this receptor encodes a presumptive transmembrane protein of 439 amino acid residues (Dembic et al. (1990) *Cytokine* 2:231; Tartaglia etal. (1992) *Immunol. Today* 13:151). The other receptor type, termed TNFR-I (Type B, Type β, 55 kDa or htr antigen) has an apparent molecular weight of about 55 kDa. The gene for this protein encodes a transmembrane protein of 426 amino acid residues (Schall et al. (1990) *Cell* 61:361; Loetscher et al. (1990) *Cell* 61:351; Tartaglia etal. (1992) *Immunol. Today* 13:151). Both receptor types show high affinity binding of either TNF-α or TNF-62 . The two receptor types are immunologically distinct but their extracellular domains show similarities in the pattern of cysteine residue locations in four domains (Dembic et al. (1990) *Cytokine* 2:231).

[0010] Soluble TNF binding proteins in human serum and urine (Seckinger et al. (1989) *J. Biol. Chem.* 264:11966; Olsson et al. (1989) *Eur. J. Haematol.* 42:270; and Engelmann et al. (1990) *J. Biol. Chem.* 265:1541) that can neutralize the biological activities of TNF-α and TNF-β have been identified. Two types have been identified and designated sTNF RI (or TNF BPI) and sTNF RIh (or TNF BPII). These soluble forms are truncated forms of the two types of TNF receptors. The soluble receptor forms apparently arise as a result of shedding of the extracellular domains of the receptors, and concentrations of about 1-2 ng/mL are found in the serum and urine of healthy subjects (Aderka et al. (1992) *Lymphokine and Cytokine Res.* 11:157; Chouaib et al. (1991) *Immunol. today* 12:141). The levels of the soluble receptors vary from individual to individual but are stable over time for given individuals (Aderka et al. (1992) *Lymphokine and Cytokine Res.* 11:157).

[0011] The physiological role of the soluble TNF receptors is not known. It is known that both types of soluble receptors can bind to TNF in vitro and inhibit its biological activity by competing with cell surface receptors for TNF binding.

Hemorrhagic Virus Desease and Disorders

[0012] A syndrome referred to as viral hemorrhagic fever is caused by one of several RNA viruses that include members of the viral families of Arenaviridae, Bunyaviridae, Filoviridae and Flaviviridae (see, e.g., Peters et al., Textbook of human virology (Belshe, ed.), Mosby Year Book, pp. 699-712 (1991)). Pronounced hemorrhage manifestations are characteristic of these fevers as well as disseminated intravascular coagulation (DIC), generalized shock, and a high mortality rate (30%-90%) (Fisher-Hoch et al., *J. Infect. Dis.*, 1523:887-894 (1985); Fisher-Hoch, *Rev. Med. Virol.*, 3:7-13 (1993); Murphy et al., Virology (Fields and Knipe, eds.), Raven, N.Y., pp. 936-942 (1990)). Despite some understanding of the progress of these diseases and responses, there are few, if any, effective treatments.

[0013] Due to the severity and breadth of viral hemorrhagic diseases and other disorders associated with a deleterious immune response, there is a great need for effective treatments of such diseases, disorders and conditions. Therefore, it is an object herein to provide treatments for such diseases and disorders.

SUMMARY OF THE INVENTION

[0014] Methods and compositions for treating disorders and diseases involving acute inflammatory responses are provided. The methods and composition provided herein are used to treat various types viral and infectious diseases and other diseases, conditions and disorders, including but are not limited to, viral hemorrhagic diseases and other acute infectious diseases, sepsis, cachexia, rheumatoid arthritis and other autoimmune disorders, acute cardiovascular events, chronic myelogenous leukemia and transplanted bone marrow-induced graft-versus-host disease, septic shock, immune complex-induced colitis, cerebrospinal fluid inflammation, autoimmune disorders, multiple sclerosis and other such disorders. Other disorders, conditions and diseases include, but are not limited to, trauma, such as polytrauma, burns, major surgery; systemic inflammatory response syndrome (SIRS); adult respiratory distress syndrome (ARDS); acute liver failure; inflammatory bowel disease, Crohn's disease and other such disorders.

[0015] In a particular embodiment, methods and compositions for treating viral and other infectious diseases, particularly bacterial sepsis and viral hemorrhagic diseases or disorders, particularly those viral hemorrhagic diseases or disorders caused by infection with a Bunyaviridaea, a Filoviridae, a Flaviviridae, or an Arenaviridae virus, and other disorders, such as sepsis, particularly that associated with exposure to gram negative bacterial endotoxins, and shock, including that associated with trauma, and infections, such as parasitic infections, that are characterized by an immunologic response, particularly an acute inflammatory responses, involving cellular activation, including production of tumor necrosis factors, interleukins, chemokines and interferons are provided.

[0016] Compositions for effecting such treatment are also provided. Tetracycline and tetracycline-like compounds and the blood-derived compositions for effecting such treatment are provided herein. It is shown herein that tetracycline compounds and tetracycline-like compounds as defined herein can be used for treatment of disorders involving acute inflammatory responses. The tetracycline and tetracycline-like compounds are used to treat the disorders and also to produce blood product compositions from donors for the treatment of the disorders. The blood product compositions and the tetracycline and tetracycline-like compounds can be used together or each can be used for treatment of these disorders.

[0017] Also provided are methods of preparing blood or fractions thereof for use in preparing compositions for

treatment of acute inflammatory conditions, disorders and diseases, by treating the blood or fraction thereof in vitro or in vivo with a compound that is tetracycline or tetracycline-like compound. Hence methods for preparation of blood-derived compositions for treatment of diseases, conditions and disorders characterized by or involving an inflammatory immune response are provided. Methods for such production are provided. The compositions are produced either in vitro or in vivo or a combination thereof by contacting blood or blood fraction or product with a tetracycline and/or tetracycline-like compound for a sufficient time to result in at least about a 3-fold increase in the level of a selected cytokine receptors, such as IL-1 receptors and/or TNF receptors. Hence, the level of receptors, such as IL-1 receptors and/or soluble TNF receptors, in the blood or blood fraction or product is tested before and after contacting with the tetracycline or tetracycline-like compound.

[0018] In particular, a method for producing a cytokine-receptor-enriched blood product by treating blood or a fraction thereof with a tetracycline or tetracycline-like compound; and harvesting, by methods described herein or known to those of skill in the art, fractions thereof, and selecting the cytokine-receptor enriched plasma, serum or other fraction. The resulting compositions are enriched for cytokine receptors compared to the blood prior to treatment. The receptors of interest include soluble tumor necrosis factor (TNF) receptors and/or interleukin-1RA (IL-1RA) receptors. Contacting the blood or fraction thereof can be effected in vitro or in vivo. Hence a method for producing cytokine-receptor-enriched compositions by treating white blood cells in vitro with a tetracycline or tetracycline-like compound to induce receptor expression; and collecting extracellular medium is provided.

[0019] The resulting compositions and use thereof for treatment of conditions, diseases and disorders associated with acute inflammatory responses are provided.

[0020] Processes for producing compositions suitable for treating viral hemorrhagic diseases or disorders are provided. These processes include some or all of the steps of: a) administering one or more tetracycline compounds to a mammal; b) collecting blood from the mammal; and c) recovering serum or plasma from the collected blood to thereby produce a composition for use in treating the disorders or diseases. Such compositions, which are preferably derived from the plasma, can be used to treat viral hemorrhagic diseases or disorders, particularly those viral hemorrhagic diseases or disorders caused by infection of a Bunyaviridae, a Filoviridae, a Flaviviridae, or an Arenaviridae virus. These compositions also can be used to treat any disorder involving a cytotoxic response, including but not limited to sepsis and endotoxic shock. The plasma (or serum portion) may be further fractionated and fractions that possess the desired therapeutic activity (treatment of symptoms associated with the viral infection, shock or other such disorder) identified empirically and formulated, if necessary, into compositions for treatment of the mammal. For humans, the plasma (or blood) is preferably derived from a human treated with a tetracycline compound.

[0021] In particular, plasma or derivatives of the plasma produced by administering a tetracycline or tetracycline-like compound, and then isolating the fraction rich in released soluble factors, such as Il-1 receptors and TNF-1 receptors. The plasma fraction is for treating acute events, including the viral infections, and cardiovascular events. Hence compositions containing these soluble receptors, immunoattenuating factors, are provided. These are produced by administering a tetracycline compound or a tetracycline-like compound to induce the factors, harvesting the plasma, optionally enriching the plasma for these factors that sop up inflammatory factors. The resulting composition is administered.

[0022] Also provided are the resulting blood-derived compositions, and methods of treatment of treating viral hemorrhagic diseases or disorders and other diseases involving a cytotoxic response in which TNF or IL-1 or both or other cytokines or receptors therefor are elevated, by administering the blood-derived compositions.

[0023] Also provided are methods of treatment of these conditions, diseases and disorders (collectively referred to as conditions). The compositions are administered to a mammal with a condition associated with or characterized by an acute inflammatory response. These compositions can be administered in combination with tetracycline and/or tetracycline-like compounds and also optionally in combination with other therapies for each disorder. The combination therapies may be administered simultaneously, consecutively, intermittently or in any desired or effective order. The may be repeated as needed.

[0024] Hence in certain embodiments, tetracycline and tetracycline-like compounds other related compounds and the blood-derived compositions provided herein are used to treat various types viral and infectious diseases, particularly viral hemorrhagic diseases or disorders, particularly those viral hemorrhagic diseases or disorders caused by infection with a Bunyaviridae, a Filoviridae, a Flaviviridae, or an Arenaviridae virus, and other disorders, such as sepsis, particularly that associated with exposure to gram negative bacterial endotoxins, and shock, including that associated with trauma, and infections, such as parasitic infections, that are characterized by an immunologic response, particularly acute inflammatory responses, involving cellular activation, including production tumor necrosis factors, interleukins, chemokines and interferons. Hence the tetracycline and tetracycline-like compounds and the blood-derived compositions provided herein are used to treat conditions and disorders, including but are not limited to, sepsis, cachexia, rheumatoid arthritis, chronic myelogenous leukemia and transplanted bone marrow-induced graft-versus-host disease, septic shock, immune complex-induced colitis and cerebrospinal fluid inflammation.

[0025] Encompassed within the methods are the uses of any tetracycline compound, or derivatives thereof, or a mixture thereof, and tetracycline-like compounds that can alleviate, reduce, ameliorate, or prevent viral hemorrhagic diseases or disorders and other acute inflammatory response; or place or maintain in a state of remission of clinical symptoms or diagnostic markers associated with such diseases or disorders.

[0026] Of particular interest are methods of treatment for viral hemorrhagic diseases and disorders caused by infection with a Bunyaviridae, a Filoviridae, a Flaviviridae, or an Arenaviridae virus. The compounds and compositions provided herein can be used alone or in combination with other treatments for hemorrhagic disorders. Viruses that cause

hemorrhagic diseases include, but are not limited to, Bunyaviridae, a Filoviridae, a Flaviviridae, and Arenaviridae viruses. The Bunyaviridae viruses include, but are not limited to, bunyavirus (Bunyamwera, Bwamba, California, Capim, Guama, phlebovirus koongol, patois, simbu and tete viruses), sandfly fever virus, Rift Valley fever virus of sheep and ruminants, Nairovirus, Crimean-Congo hemorrhagic fever virus, Uukuvirus, Uukuniemi virus, Hantaan virus and Korean hemorrhagic fever virus. In particular, the Bunyaviridae viruses include, Crimean-Congo hemorrhagic fever virus, Hantaan virus and Korean hemorrhagic fever virus. The Filoviridae viruses include, but are not limited to, ebola virus, such as the Zaire, Sudan, Reston and Ivory Coast subtypes, and Marburg viruses. Other Flaviviridae virus include flavivirus, Brazilian encephalitis virus, Bussuquara virus, Dengue virus, iiheus virus, Israel turkey meningoencephalitis virus, Japanese B encephalitis virus, Kunjin virus, Kyasanur forest disease virus, Langat virus, Louping ill virus, Modoc virus, Murray valley encephalitis virus, Ntaya virus, omsk hemorrhagic fever virus, powassan virus, St. Louis encephalitis virus, spondwnei virus, tick-borne encephalitis, Uganda S virus, US bat salivary gland virus, wesselsbron virus, West Nile fever virus, yellow fever virus, Zika virus, European tick-borne encephalitis, Far Eastern tick-borne encephalitis virus, Russian tick-borne encephalitis, and Dengue virus, including but are not limited to, Dengue type 1, Dengue type 2, Dengue type 3 and Dengue type 4 virus. The Arenaviridae viruses include, but are not limited to, Junin virus, Lassa virus such as the Josiah strain or Nigerian strain, Machupo virus, Pichinde virus, lymphocytic choriomeningitis virus, Lassa fever virus and arenavirus.

[0027] Provided herein are combinations, preferably in the form of pharmaceutical compositions, including one or more tetracycline compound(s) and one or more anti-hemorrhagic virus treatments. The combinations are typically pharmaceutical compositions that include a tetracycline compound formulated for single dosage administration, and an agent, other than a tetracycline compound, that is an anti-hemorrhagic viral agent, such as a vaccine, antibody or other pharmaceutical. The compound and agent can be administered separately, such as sequentially, or can be administered intermittently, or together as two separate compositions or as a mixture in a single composition. The dosage of each can be empirically determined, but is generally the dosage of a agent normally used to treat the hemorrhagic viral infection, and an amount of a tetracycline compound sufficient to further enhance treatment, or sufficient when used alone to reduce or ameliorate or in some manner reduce symptoms. The combinations can be packaged as kits.

[0028] In a preferred embodiment, the combination contains a single composition containing the tetracycline compound and anti-hemorrhagic virus agent formulated for oral delivery or two compositions, one containing a tetracycline compound and the other an anti-viral-hemorrhagic agent, where each is in a pharmaceutically acceptable carrier or excipient in tablet, capsule, or other single unit dosage form. Alternatively, the two components can be mixed in a single composition. In other embodiments, the compositions are formulated for rectal, topical, inhalation, buccal (e.g., sublingual), parenteral (e.g., subcutaneous, intramuscular, intradermal, or intravenous including bolus injection) and transdermal administration. Specific therapeutic regimens, pharmaceutical compositions, and kits are also provided.

[0029] Also provided is a method for treating viral hemorrhagic diseases or disorders in mammals, including humans, particularly those viral hemorrhagic diseases or disorders caused by infection of any virus causing such disease or disorder, including but not limited to a Bunyaviridae, a Filoviridae, a Flaviviridae, or an Arenaviridae virus, by administrating a therapeutically effective and non-lethal amount of one or more tetracycline compound(s).

[0030] Tetracycline compounds include, but are not limited to chlortetracycline, demeclocycline, doxycycline, methacycline, minocycline, oxytetracycline and tetracycline. Tetracycline-like compounds are those that share the property of altering folic acid metabolism in bacteria. Such compounds include thalidomide and sulfa drugs.

[0031] Anti-hemorrhagic virus treatments include treatment protocols and agents that are used to treat hemorrhagic viral diseases or ameliorate the symptoms thereof. Such agents include, but are not limited to agents that inhibit interleukin-1 (IL-1) and agents that inhibit TNF. Other anti-hemorrhagic viral agents, include, but are not limited to, anti-viral vaccines, anti-viral antibodies, a viral-activated immune cells, such as activated cytotoxic cells, and viral-activated immune serum.

[0032] Agents that inhibit IL-1, include, but are not limited to, anti-IL-1 antibodies, anti-IL-1 receptor antibodies, IL-1 receptor antagonists, an IL-1 production inhibitors, IL-1 receptor production inhibitors, and IL-1 releasing inhibitors.

[0033] IL-1 receptor antagonists include, but are not limited to, the IL-1 receptor antagonist (IL-1Ra), IL-1 receptor intracellular ligand protein, a Type II IL-1 receptor, a soluble IL-1 receptor, a non-biologically active (i.e., non-functional) mutein of IL-1 that binds to IL-receptors, a non-functional mutein of IL-1 receptor and small molecule antagonists, such as histamine antagonist, a aryl-or heteroaryl-1-alkyl-pyrrole-2-carboxylic acid compound and a 5-lipoxygenase pathway inhibitor.

[0034] IL-1 production inhibitors include antisense oligonucleotides, 5-hydroxy- and 5-methoxy-2-amino-pyrimidines, a 3-substituted-2-oxindole-1-carboxamide, a 4,5-diaryl-2(substituted)imidazole and a 2-2'-[1,3-propan-2-onediyl-bis(thio)]bis-1-H-imidazole. IL-1 releasing inhibitors include IL-1 converting enzyme inhibitors, such as, but are not limited to, a peptide based interleukin-1 beta converting enzyme inhibitor, a pyridazinodiazepine, SDZ 224-015, an aspartate-based inhibitor, an aspartyl alpha-((1-phenyl-3-(trifluoromethyl)-pyrazol-5-yl)oxy)methyl ketone, L-741,494, TX, CPP-32 and CMH-1.

[0035] Agents that inhibit TNF include, but are not limited to, anti-TNF antibody (polyclonal or monoclonal), an anti-TNF receptor antibody (polyclonal or monoclonal), a TNF receptor antagonist, a TNF production inhibitor, a TNF receptor production inhibitor and a TNF releasing inhibitor. Anti-TNF monoclonal antibodies, include, but are not limited to, Mabp55r, Mabp75r, 3B10, h3B10-9, MAK 195F, CA2 and CDP571. Other TNF receptor antagonists include, but are not limited to, soluble TNF receptor, a non-functional mutein that binds to the TNF receptor, but does not exhibit TNF biological activity, a non-functional mutein of TNF and small molecule antagonists, such as but are not limited to, a mercapto alkyl peptidyl compound, an arylsul-

fonyl hydroxamic acid derivative, a salt of an alkaline-earth metal, a pentoxifylline, a hydroxamic acid compound, a retinoic acid, a histamine antagonist, a leflunomide, a 1-Alkoxy-2-(alkoxy- or cycloalkoxy-)-4-(cyclothioalkyl- or cyclothio-alkenyl-)benzene, a vinigrol, a cyclohexene-ylidene derivative, a quinazoline compound and BN 50739. Other TNF receptor antagonists include, but are not limited to, TNF receptor death domain ligand protein, a tumor necrosis factor binding protein (TNF-BP), a TNF receptor-IgG heavy chain chimeric protein, a bacterial lipopolysaccharide binding peptide derived from CAP37 protein and a Myxoma virus T2 protein. TNF production inhibitors, include antisense oligonucleotides, quinoline-3-carboxamide compounds and derivatives of 2-pyrrolidinone. TNF releasing inhibitors include isoxazoline compounds and catechol diether compounds.

[0036] Methods herein are for stimulating release of the receptors such as, but not limited to, TNF-α, IL-1 receptors and other soluble factors that down-regulate excessive T-helper 1 (TH1) response, that is stimulated by tetracycline administration. The receptors are those that bind to and/or inhibit inflammatory factors that are released in various inflammatory conditions, viral infections, bacterial infections, and conditions associate with fungal and parastitic infections, inflammatory responses, such as asthma, sepsis, rheumatoids, atherosclerosis, inflammatory responses associated with injury, and cardiovascular events and events related to cell activation, i.e., acute events brought on by excessive release of inflammatory factors.

DETAILED DESCRIPTION OF THE INVENTION

[0037] Particular compositions, combinations, kits and methods are described in the sections and subsections as follows:

[0038] A. Definitions

[0039] B. Combinations and Kits and Compositions for Treatment of Acute Inflammatory Responses

[0040] 1. Tetracycline-like compounds

[0041] 2. Tetracycline compounds

[0042] a. Anti-inflammatory activity of tetracyclines

[0043] b. Exemplary tetracycline compounds

[0044] (1) Chlortetracycline

[0045] (2) Demeclocycline

[0046] (3) Doxycycline

[0047] (4) Methacycline

[0048] (5) Minocycline

[0049] (6) Oxytetracycline

[0050] (7) Tetracycline

[0051] (8) Other Chemically-Modified Tetracyclines

[0052] C. Hemorrhagic Viruses and the Immune Response

[0053] D. Parmaceutical Compositions, Formulation and Modes of Administration thereof

[0054] 1. Anti-viral-hemorrhagic agents

[0055] a. Interleukin-1 (Il-1) inhibitors

[0056] b. Tumor necrosis factor (TNF) inhibitors

[0057] c. Anti-viral vaccine, antibody and virally-activated immune cells and serum

[0058] (1) Anti-viral vaccine

[0059] (a) Anti-Bunyaviridae Vaccine

[0060] (b) Anti-Filoviridae Vaccine

[0061] (c) Anti-Flaviviridae Vaccine

[0062] (d) Anti-Arenaviridae Vaccine

[0063] (2) Anti-viral antibodies

[0064] (a) Anti-Bunyaviridae Antibody

[0065] (b) Anti-Filoviridae Antibody

[0066] (c) Anti-Flaviviridae Antibody

[0067] (d) Anti-Arenaviridae Antibody

[0068] (3) Viral-activated immune cell and serum

[0069] (4) Small molecule anti-viral agents

[0070] 2. Formulation and routes of administration

[0071] E. Blood-derived Compostions and Methods of Treatment

[0072] 1. Blood-derived compositions and processes for producing compositions for treating diseases and disorders characterized by or associated with acute inflammatory responses

[0073] a. Preparation of Serum and Plasma

[0074] b. Further Fractionation of Plasma

[0075] (1) Preparation of Albumin-Containing Fraction

[0076] (2) Preparaton of Globulin-Containing Fraction

[0077] (3) Preparation of AHF-Containing Fraction

[0078] (4) Preparation of Fraction Containing Soluble IL-1 Receptor or Soluble TNF Receptor

[0079] C. Methods of treatment using the resulting blood-derived compositions

[0080] F. Viral hemorrhagic Disease or Disorder and Diagnosis Thereof

[0081] 1. Bunyaviridae Virus Infection

[0082] 2. Filoviridae Virus Infection

[0083] 3. Flaviviridae Virus Infection

[0084] 4. Arenaviridae Virus Infection

[0085] G. Examples

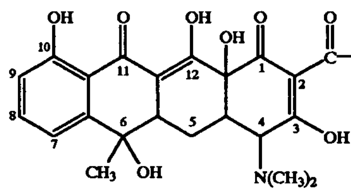
[0086] A. Definitions

[0087] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is com-

monly understood by one of skill in the art to which this invention belongs. Where permitted, all patents, applications, published applications and other publications and sequences from GenBank and other data bases referred to throughout the disclosure herein are incorporated by reference in their entirety.

[0088] As used herein, "tetracycline compound" refers to any compound having the activity of a tetracycline, prodrugs, salts, esters or other derivatives of tetracycline, preferably in a pharmaceutically acceptable form, known to those of skill in the art.

[0089] Tetracycline, which is well known to those of skill in the art, has the structure:



[0090] It is intended herein for the the term "tetracycline" to encompass all pharmaceutically active species of tetracycline compounds, solutions thereof and mixtures thereof, prodrugs thereof and any drug recognized as a tetracycline. Tetracycline includes forms, such as hydrated forms, and compositions such as aqueous solutions, hydrolyzed products or ionized products of these compounds; and these compounds may contain different numbers of attached water molecules. Thus, as used herein, the term tetracycline compound encompasses all derivatives and analogs and modified forms thereof, including but not limited to, those set forth herein. Tetracycline and tetracycline-like compounds include, but are not limited to aspirin, aureomycin, apicycline, chlortetracycline, clomocycline, demeclocyline, guamecycline, lymecycline, meclocycline, methacycline, minocycline, oxytetracycline, penimepicycline, pipacycline, rolitetracycline, sancycline, and senociclin, as well as any others falling within the above formula. Also included among tetracycline-like compounds are compounds that alter bacterial folic acid metabolism, such as sulfa drugs, including sulfonamides, and thalidomide. Such compounds can be identified by their ability to alter bacterial folic acid metabolism.

[0091] As used herein, tetracycline-like compounds, such as aureomycin, sulfa drugs and thalidomide, refer to compounds that have the activity of tetracycline in the methods herein. Such compounds can be identified by their ability to alter folic acid metabolism in bacterial species, particularly those in which tetracycline alters folic acid metabolism.

[0092] As shown herein, a tetracycline and tetracycline-like compound herein is a compound that stimulates release of soluble factors in the blood that attenuate inflammatory responses.

[0093] Any tetracycline compound(s), when used alone or in combination with other compounds, that can alleviate, reduce, ameliorate, prevent, or place or maintain in a state of remission of clinical symptoms or diagnostic markers associated with viral hemorrhagic diseases or disorders, particularly those viral hemorrhagic diseases or disorders caused by infection of a Bunyaviridae, a Filoviridae, a Flaviviridae, or an Arenaviridae virus, are intended for use in the methods, compositions and combinations provided herein.

[0094] As used herein, an anti-hemorrhagic virus treatment refers to any treatment designed to treat hemorrhagic viral infections by lessening or ameliorating the symptoms. Treatments that prevent the infection or lessen its severity are also contemplated. An anti-hemorrhagic virus agent (used interchangeable with "anti-viral-hemorrhagic agent") refers to any agents used in the treatment. These include any agents, when used alone or in combination with other compounds, that can alleviate, reduce, ameliorate, prevent, or place or maintain in a state of remission of clinical symptoms or diagnostic markers associated with viral hemorrhagic diseases or disorders, particularly those viral hemorrhagic diseases or disorders caused by infection of a Bunyaviridae, a Filoviridae, a Flaviviridae, or an Arenaviridae virus, can be used in methods, combinations and compositions provided herein. Non-limiting examples of anti-viral-hemorrhagic agents include interleukin-1 (IL-1) inhibitors, tumor necrosis factor (TNF) inhibitors, anti-viral vaccines, anti-viral antibodies, viral-activated immune cells and viral-activated immune sera.

[0095] As used herein, anti-hemorrhagic virus agent (anti-viral-hemorrhagic agent) or anti-hemorrhagic virus treatment does not encompass "tetracycline compound" or use thereof for treatment, but encompasses all agents and treatment modalities known to those of skill in the art to ameliorate the symptoms of a hemorrhagic viral infection.

[0096] As used herein, a cytokine is a factor, such as lymphokine or monokine, that is produced by cells that affect the same or other cells. A "cytokine" is one of the group of molecules involved in signaling between cells during immune responses. Cytokines are proteins or peptides; and some are glycoproteins.

[0097] As used herein, "interleukin (IL)" refers to a large group of cytokines produced mainly by T cells, although some are also produced by mononuclear phagocytes, or by tissue cells. They have a variety of functions, but most of them are involved in directing other cells to divide and differentiate. Each interleukin acts on specific, limited groups of cells which express the correct receptors for that cytokine.

[0098] As used herein, "interleukin-1 (IL-1)" refers to interleukins made by certain antigen presenting cells (APCs) that, along with IL-6, act as co-stimulatory signals for T cell activation. The IL-1 gene family includes IL-1α, IL-1β and IL-1 receptor antagonist (IL-1Ra) (Dinarello, *Eur. Cytokine Netw.,* 5(6):517-522 (1994)). Each member is first synthesized as a precursor protein; the precursors for IL-1 (proIL-1α and proIL-1β) have molecular weights of about 31,000 Da. The proIL-1a and mature 17,000 Da IL-1α are biologically active whereas the proIL-1β requires cleavage to a 17,000 Da peptide for optimal biological activity. The IL-IRa precursor has a leader sequence and is cleaved to its mature form and secreted like most proteins. IL-1α and IL-1β are potent agonists where IL-1Ra is a specific receptor antagonist. Moreover, IL-IRa appears to be a pure receptor antagonist with no agonist activity in vitro or in vivo. Although IL-1Ra is a secreted protein, there is another form

of this molecule which is retained inside cells. It is called "intracellular" (ic) IL-1Ra. IcIL-1Ra results from alternate mRNA splice insertion of the IL-1Ra gene replacing the exon coding for the signal peptide. The forms of IL-1Ra are functionally indistinguishable.

[0099] Thus, reference, for example, to "IL-1" encompasses all proteins encoded by the IL-1 gene family including IL-1α, IL-1β, IL-1Ra and icIL-lRa, or an equivalent molecule obtained from any other source or that has been prepared synthetically. It is intended to encompass IL-1 with conservative amino acid substitutions that do not substantially alter its activity. Suitable conservative substitutions of amino acids are known to those of skill in this art and may be made generally without altering the biological activity of the resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, e.g., Watson et al. *Molecular Biology of the Gene,* 4th Edition, 1987, The Bejacmin/Cummings Pub. co., p.224).

[0100] Such substitutions are preferably made in accordance with those set forth in TABLE 1 as follows:

TABLE 1

Original residue	Conservative substitution
Ala (A)	Gly; Ser
Arg (R)	Lys
Asn (N)	Gln; His
Cys (C)	Ser
Gln (Q)	Asn
Glu (E)	Asp
Gly (G)	Ala; Pro
His (H)	Asn; Gln
Ile (I)	Leu; Val
Leu (L)	Ile; Val
Lys (K)	Arg; Gln; Glu
Met (M)	Leu; Tyr; Ile
Phe (F)	Met; Leu; Tyr
Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr
Tyr (Y)	Trp; Phe
Val (V)	Ile; Leu

[0101] Other substitutions are also permissible and may be determined empirically or in accord with known conservative substitutions.

[0102] As used herein, the amino acids, which occur in the various amino acid sequences appearing herein, are identified according to their well-known, three-letter or one-letter abbreviations. The nucleotides, which occur in the various DNA fragments, are designated with the standard single-letter designations used routinely in the art.

[0103] As used herein, "IL-1 inhibitor" encompasses any substances that prevent or decrease production, post-translational modification(s), maturation, or release of IL-1, or any substances that interfere with or decrease the efficacy of the interaction between IL-1 (see, e.g., SEQ ID Nos. 1 and 2) and IL-1 receptor (see, e.g., SEQ ID Nos. 3 and 4). Preferably, the IL-1 inhibitor is an anti-IL-1 antibody, an anti-IL-1 receptor antibody, an IL-1 receptor antagonist, an IL-1 production inhibitor, an IL-1 receptor production inhibitor and an IL-1 releasing inhibitor.

[0104] As used herein, the terms "a therapeutic agent", "therapeutic regimen", "radioprotectant", "chemotherapeutic" mean conventional drugs and drug therapies, including vaccines, which are known to those skilled in the art. "Radiotherapeutic" agents are well known in the art.

[0105] As used herein, "interleukin-1 converting enzyme (ICE)" refers to a protease that processes the IL-1β precursor (pIL-1β) to the mature IL-1β (mIL-1β) (U.S. Pat. No. 5,552,536). ICE generates fully active mIL-1i by cleaving pIL-1β between Asp_{116} and Ala_{117}, a unique site for pheromone processing. The sequence around this cleavage site, -Tyr-Val-His-Asp-Ala-, is evolutionarily conserved in all known pIL-1β polypeptides. Active human ICE is a heterodimer with a 1:1 stoichiometric complex of p20 and p10 subunits. Cloned cDNA have revealed that ICE is constitutively expressed as a 45 kDa proenzyme (p45) composed of a 14 kDa prodomain, followed by p20 which contains the active site CyS_{285}, a 19 residue connecting peptide that is not present in the mature enzyme, and p10, a required component of the active enzyme. The mature subunits are flanked by Asp-X sequences. Mutational analysis of these sites and expression in heterologous systems indicates that the generation of active enzyme is autocatalytic. Murine and rat ICE have also been cloned and show a high degree of sequence similarity including these structural motifs.

[0106] As used herein, "tumor necrosis factor (TNF)" refers to a group of proinflammatory cytokines encoded within the major histocompatibility complex (MHC). TNF family members include TNFα (also known as cachectin) and TNFβ (also known as lymphotoxin). Complementary cDNA clones encoding TNFα (Pennica et al., *Nature,* 312:724 (1984)) and TNFβ (Gray et al., *Nature,* 312:721 (1984)) have been isolated. Therefore, reference, for example, to "TNF" encompasses all proteins encoded by the TNF gene family including TNFα and TNFβ, or an equivalent molecule obtained from any other source or that has been prepared synthetically. It is intended to encompass TNF with conservative amino acid substitutions that do not substantially alter its activity.

[0107] As used herein, "TNF inhibitor" encompasses any substances that prevent or decrease production, post-translational modification(s), maturation, or release of TNF, or any substances that interfere with or decrease the efficacy of the interaction between TNF (see, e.g., SEQ ID Nos. 14 and 15) and TNF receptor (see, SEQ ID Nos. 16 and 17). Preferably, the TNF inhibitor is an anti-TNF antibody, an anti-TNF receptor antibody, a TNF receptor antagonist, a TNF production inhibitor, a TNF receptor production inhibitor and a TNF releasing inhibitor.

[0108] Native TNF receptors are characterized by distinct extracellular, transmembrane and intracellular domains. Two distinct TNF receptors of about 55 kd ("TNF-R1") and about 75 kDa ("TNF-R2") have been identified. Numerous studies have demonstrated that TNF-R1 is the receptor which signals the majority of the pleiotropic activities of TNF. The domain required for signaling cytotoxicity and other TNF-mediated responses has been mapped to the about 80 amino acids near the C-terminus of TNF-R1. This domain is therefore termed the "death domain" ("TNF-R death domain" and "TNF-R1-DD") (see, U.S. Pat. No. 5,852,173; and Tartaglia et al., *Cell,* 74:845-853 (1993)).

[0109] As used herein, "antisense polynucleotides" refer to synthetic sequences of nucleotide bases complementary to

mRNA or the sense strand of double stranded DNA. A mixture of sense and antisense polynucleotides under appropriate conditions leads to the binding of the two molecules, or hybridization. When these polynucleotides bind to (hybridize with) mRNA, inhibition of protein synthesis (translation) occurs. When these polynucleotides bind to double stranded DNA, inhibition of RNA synthesis (transcription) occurs. The resulting inhibition of translation and/or transcription leads to an inhibition of the synthesis of the protein encoded by the sense strand.

[0110] As used herein, an antisense oligonucleotide that contains a sufficient number of nucleotides to inhibit translation of an mRNA, such as an interleukin-1 (IL-1), such as IL-1α, or TNF. An antisense oligonucleotide refers to any oligomer that prevents production or expression of, for example, IL-1 polypeptide. The size of such an oligomer can be any length that is effective for this purpose. In general, the antisense oligomer is prepared in accordance with the nucleotide sequence of a portion of the transcript of interest (i.e., IL-1 and TNF) that includes the translation initiation codon and contains a sufficient number of complementary nucleotides to block translation.

[0111] As used herein, "vaccine" refers to any composition for active immunological prophylaxis. A vaccine may be used therapeutically to treat a disease, or to prevent development of a disease or to decrease the severity of a disease either proactively or after infection. Non-limiting examples of vaccines include, but are not limited to, preparations of killed microbes of virulent strains or living microbes of attenuated (variant or mutant) strains, or microbial, fungal, plant, protozoa, or metazoa derivatives or products. "Vaccine" also encompasses protein/peptide and nucleotide based vaccines.

[0112] As used herein, "cytotoxic cells" refers to cells that kill virally infected targets expressing antigenic peptides presented by MHC class I molecules.

[0113] As used herein, "treating hemorrhagic viral diseases or disorders" means that the diseases and the symptoms associated with the hemorrhagic viral diseases or disorders are alleviated, reduced, ameliorated, prevented, placed in a state of remission, or maintained in a state of remission. Additionally, as used herein, "a method for treating hemorrhagic viral diseases or disorders" means that the hallmarks of hemorrhagic viral diseases or disorders are eliminated, reduced or prevented by the treatment. Non-limiting examples of the hallmarks of the viral hemorrhagic diseases or disorders include disseminated intravascular coagulation (DIC), generalized shock, and the highest mortality rate (30%-90%).

[0114] As used herein, a blood-derived composition (or immune composition) refers to the composition produced from the blood of mammals treated with a tetracycline and/or tetracycline-like compound. It also refers to the compositions produced by in vitro treatment of blood or a blood fraction with a tetracycline or tetracycline-like compound. These blood-derived compositions are for treating, not only the hemorrhagic disorders, but also for alleviating any disorder involving a deleterious immune response, such as septic shock and endotoxic shock.

[0115] The immune response to certain infectious agents, such as viruses, parasites and bacteria, and in certain diseases and conditions, activate cells and products thereof that have deleterious consequences. For example, LPS (lipopolysaccharide) binds to immunoglobin M and this complex activates the complement system with the release of C3b, which material in turn activates the polymorphonuclear leukocytes (PMN), monocytes, neutrophils, macrophage and endothelial cells. The activation of these substances stimulates the release of several mediators of septic shock including tumor necrosis factor (TNF-a) interleukin-1 (IL-1) and other interleukins including IL-6 and IL-8, platelet-activating factor (PAF), prostaglandins and leukotrienes (see, e.g., (1991) *Ann. Intern. Med.* 115: 464-466 for a more comprehensive listing). Of these, the two cytokines TNF-α and IL-1 lead to many of the physiologic changes which eventuate into septic shock.

[0116] As used herein, an acute inflammatory disease, condition or disorder, refers to any condition, disease or disorder in which a deleterious elevation of cytokines and other inflammatory mediators occurs. For purposes herein, disease, condition and disorder refer to the manifestation of such elevation. In general a disease is caused by an infectious agent, a disorder refers to a disease that does not have a known infectious agent as a cause and a condition is used to capture all such symptoms and characteristics associated with acute inflammatory responses. They are referred to herein in the alternative to ensure that all are encompassed.

[0117] As used herein, "serum" refers to the fluid portion of the blood obtained after removal of the fibrin clot and blood cells, distinguished from the plasma in circulating blood.

[0118] As used herein, "plasma" refers to the fluid, non-cellular portion of the blood, distinguished from the serum obtained after coagulation.

[0119] As used herein, "albumin" refers to a type of protein, varieties of which are widely distributed throughout the tissues and fluids of plants and animals, especially animal blood. Albumin are soluble in pure water, precipitable from solution by strong acids and coagulable by heat in acid or neutral solution.

[0120] As used herein, "globulin" refers to a family of proteins precipitated from plasma (or serum) by half-saturation with ammonium sulfate. Globulin may be further fractionated by solubility, electrophoresis, ultracentrifugation, and other separation methods into many subgroups, the main groups being α-, β-, and γ-globulins.

[0121] As used herein, "antihemophilic factor (AHF)" refers the fraction of blood that contains Factor VIII and/or von Willebrand's factor, which are important in the blood clotting mechanism (see, e.g., U.S. Pat. No. 4,435,318). Factor VIII serves as a co-factor along with calcium and phospholipid to enable Factor IX_a to cleave zymogen Factor X to thus activate Factor X, all being a part of the complex coagulation cascade system. Von Willebrand's factor (vWF) apparently acts in the aggregation of platelets which provide the necessary phospholipid. The absence of either of these factors may result in prolonged bleeding times. Factor V also serves an important role in the coagulation system by aiding activated Factor X in the cleavage of prothrombin to thrombin. (The Plasma Proteins, Vol. III, 2nd Ed., Structure, Function, Genetic Control (1977) (Academic Press, Inc., N.Y.) p. 422-544.)

[0122] As used herein, an effective amount of a compound for treating a particular disease is an amount that is sufficient to ameliorate, or in some manner reduce the symptoms associated with the disease. Such amount may be administered as a single dosage or may be administered according to a regimen, whereby it is effective. The amount may cure the disease but, typically, is administered in order to ameliorate the symptoms of the disease. Repeated administration may be required to achieve the desired amelioration of symptoms.

[0123] As used herein, pharmaceutically acceptable salts, esters or other derivatives of the conjugates include any salts, esters or derivatives that may be readily prepared by those of skill in this art using known methods for such derivatization and that produce compounds that may be administered to animals or humans without substantial toxic effects and that either are pharmaceutically active or are prodrugs.

[0124] As used herein, treatment means any manner in which the symptoms of a condition, disorder or disease are ameliorated or otherwise beneficially altered. Treatment also encompasses any pharmaceutical use of the compositions herein.

[0125] As used herein, amelioration of the symptoms of a particular disorder by administration of a particular pharmaceutical composition refers to any lessening, whether permanent or temporary, lasting or transient that can be attributed to or associated with administration of the composition.

[0126] As used herein, substantially pure means sufficiently homogeneous to appear free of readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis and high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially chemically pure compounds are known to those of skill in the art. A substantially chemically pure compound may, however, be a mixture of stereoisomers or isomers. In such instances, further purification might increase the specific activity of the compound.

[0127] As used herein, a prodrug is a compound that, upon in vivo administration, is metabolized or otherwise converted to the biologically, pharmaceutically or therapeutically active form of the compound. To produce a prodrug, the pharmaceutically active compound is modified such that the active compound will be regenerated by metabolic processes. The prodrug may be designed to alter the metabolic stability or the transport characteristics of a drug, to mask side effects or toxicity, to improve the flavor of a drug or to alter other characteristics or properties of a drug. By virtue of knowledge of pharmacodynamic processes and drug metabolism in vivo, those of skill in this art, once a pharmaceutically active compound is known, can design prodrugs of the compound (see, e.g., Nogrady (1985) *Medicinal Chemistry A Biochemical Approach*, Oxford University Press, New York, pages 388-392).

[0128] As used herein, biological activity refers to the in vivo activities of a compound or physiological responses that result upon in vivo administration of a compound, composition or other mixture. Biological activity, thus, encompasses therapeutic effects and pharmaceutical activity of such compounds, compositions and mixtures. Biological activities may be observed in in vitro systems designed to test or use such activities. Thus, for purposes herein, the biological activity of a luciferase is its oxygenase activity whereby, upon oxidation of a substrate, light is produced.

[0129] As used herein, a receptor refers to a molecule that has an affinity for a given ligand. Receptors may be naturally-occurring or synthetic molecules. Receptors may also be referred to in the art as anti-ligands. As used herein, the receptor and anti-ligand are interchangeable. Receptors can be used in their unaltered state or as aggregates with other species. Receptors may be attached, covalently or noncovalently, or in physical contact with, to a binding member, either directly or indirectly via a specific binding substance or linker. Examples of receptors, include, but are not limited to: antibodies, cell membrane receptors surface receptors and internalizing receptors, monoclonal antibodies and antisera reactive with specific antigenic determinants [such as on viruses, cells, or other materials], drugs, polynucleotides, nucleic acids, peptides, cofactors, lectins, sugars, polysaccharides, cells, cellular membranes, and organelles.

[0130] Examples of receptors and applications using such receptors, include but are not restricted to:

[0131] a) enzymes: specific transport proteins or enzymes essential to survival of microorganisms, which could serve as targets for antibiotic [ligand] selection;

[0132] b) antibodies: identification of a ligand-binding site on the antibody molecule that combines with the epitope of an antigen of interest may be investigated; determination of a sequence that mimics an antigenic epitope may lead to the development of vaccines of which the immunogen is based on one or more of such sequences or lead to the development of related diagnostic agents or compounds useful in therapeutic treatments such as for auto-immune diseases

[0133] c) nucleic acids: identification of ligand, such as protein or RNA, binding sites;

[0134] d) catalytic polypeptides: polymers, preferably polypeptides, that are capable of promoting a chemical reaction involving the conversion of one or more reactants to one or more products; such polypeptides generally include a binding site specific for at least one reactant or reaction intermediate and an active functionality proximate to the binding site, in which the functionality is capable of chemically modifying the bound reactant [see, eg., U.S. Pat. No. 5,215,899];

[0135] e) hormone receptors: determination of the ligands that bind with high affinity to a receptor is useful in the development of hormone replacement therapies; for example, identification of ligands that bind to such receptors may lead to the development of drugs to control blood pressure; and

[0136] f) opiate receptors: determination of ligands that bind to the opiate receptors in the brain is useful

in the development of less-addictive replacements for morphine and related drugs.

[0137] As used herein, antibody includes antibody fragments, such as Fab fragments, which are composed of a light chain and the variable region of a heavy chain.

[0138] As used herein, humanized antibodies refer to antibodies that are modified to include "human" sequences of amino acids so that administration to a human will not provoke an immune response. Methods for preparation of such antibodies are known. For example, the hybridoma that expresses the monoclonal antibody is altered by recombinant DNA techniques to express an antibody in which the amino acid composition of the non-variable regions is based on human antibodies. Computer programs have been designed to identify such regions.

[0139] As used herein, production by recombinant means by using recombinant DNA methods means the use of the well known methods of molecular biology for expressing proteins encoded by cloned DNA.

[0140] As used herein, substantially identical to a product means sufficiently similar so that the property of interest is sufficiently unchanged so that the substantially identical product can be used in place of the product.

[0141] As used herein, equivalent, when referring to two sequences of nucleic acids means that the two sequences in question encode the same sequence of amino acids or equivalent proteins. When "equivalent" is used in referring to two proteins or peptides, it means that the two proteins or peptides have substantially the same amino acid sequence with only conservative amino acid substitutions (see, eg., Table 1, above)that do not substantially alter the activity or function of the protein or peptide. When "equivalent" refers to a property, the property does not need to be present to the same extent [eg., two peptides can exhibit different rates of the same type of enzymatic activity], but the activities are preferably substantially the same. "Complementary," when referring to two nucleotide sequences, means that the two sequences of nucleotides are capable of hybridizing, preferably with less than 25%, more preferably with less than 15%, even more preferably with less than 5%, most preferably with no mismatches between opposed nucleotides. Preferably the two molecules will hybridize under conditions of high stringency.

[0142] As used herein: stringency of hybridization in determining percentage mismatch is as follows:

[0143] 1) high stringency: 0.1×SSPE, 0.1% SDS, 65° C.

[0144] 2) medium stringency: 0.2×SSPE, 0.1% SDS, 50° C.

[0145] 3) low stringency: 1.0×SSPE, 0.1% SDS, 50° C. It is understood that equivalent stringencies may be achieved using alternative buffers, salts and temperatures.

[0146] The term "substantially" identical or homologous or similar varies with the context as understood by those skilled in the relevant art and generally means at least 70%, preferably means at least 80%, more preferably at least 90%, and most preferably at least 95% identity.

[0147] As used herein, a composition refers to a any mixture. It may be a solution, a suspension, liquid, powder, a paste, aqueous, non-aqueous or any combination thereof.

[0148] As used herein, a combination refers to any association between two or among more items.

[0149] As used herein, fluid refers to any composition that can flow. Fluids thus encompass compositions that are in the form of semi-solids, pastes, solutions, aqueous mixtures, gels, lotions, creams and other such compositions.

[0150] As used herein, the abbreviations for any protective groups, amino acids and other compounds, are, unless indicated otherwise, in accord with their common usage, recognized abbreviations, or the IUPAC-IUB Commission on Biochemical Nomenclature (see, (1972) *Biochem.* 11:1726).

[0151] For clarity of disclosure, and not by way of limitation, the detailed description is divided into the subsections that follow. The description below is exemplified by reference to viral hemorrhagic diseases. It is understood that the methods, compositions, combinations and kits provided and described herein may be used for treatment of any disorder, disease or condition characterized by a deleterious immune response, particularly, but not limited to, those specificed herein. Such diseases, conditions and disorders include, but are not limited to: viral infections, such as viral hemorrhagic infections, lentivirus infections, HIV infections, herpes virus infections; bacterial infections, particularly infection with pathogenic strains of *E. coli* and Streptococcus; viruses associated with sleep disorders, such as HIV; parasitic infections, such as malaria; autoimmune diseases, such a thyroid diseases, rheumatoid arthritis, and lupis; sepsis; cachexia, such as the wasting associated with HIV infection and cancer; rheumatoid arthritis; chronic myelogenous leukemia and transplanted bone marrow-induced graft-versus-host disease; septic shock; immune complex-induced colitis; cerebrospinal fluid inflammation; endotoxemia; autoimmune disorders; multiple sclerosis; cell death associated with apoptosis; thyroid diseases and other endocrine disorders in which TNF or IL-1 is implicated or is a mediator; gynecological disorders, including endometriosis and infections associated therewith; and other diseases mediated by or associated with IL-1 and/or TNF. It is also understood that IL-1 and TNF expression serve as markers for these disorders and to monitor the treatments herein and the blood compositions herein, but that these inflammatory response compounds are not necessarily the only agents involved.

[0152] B. Combinations and Kits and Compostions for Treatment of Acute Inflammatory Responses

[0153] Combinations of therapeutic agents and also compositions for treatment of acute inflammatory responses are provided herein. Several embodiments are provided.

[0154] In one embodiment, blood-derived compositions, described below, are provided. These compositions are produced by contacting mammalian blood or a fraction thereof, in vitro or in vivo, with one or more tetracycline and/or tetracycline-like compounds, as defined herein, to induce a response that is assessed by monitoring the increase in level of TNF receptors and/or IL-1 receptors. The amount of compound contacted with the blood and time of contact is sufficient to induce at least a three-fold increase from

baseline, which is variable from individual-to-individual and species-to-species, of TNF and/or IL-1 receptors. The total increase of either must be at least about three-fold to ensure a sufficient concentration of the receptors and other factors in the blood or fraction thereof. The resulting blood or fraction thereof can be further fractionated, such that the selected fraction retains the activity of the original blood, such as against hemorrhagic and inflammatory factors, and is then administered to a recipient mammal, that is preferably species and blood type matched to the donated blood or fraction. The blood or fraction thereof can be stored, preferably at about −70° C. or under other conditions appropriate for storage of blood products, but is preferably not freeze-dried.

[0155] The blood product may also be administered to the recipient in combination with a tetracycline and/or tetracycline-like compound. Such administration can be simultaneous or sequential. If administered separately they should be administered within 24 hours, preferably within 6 hours. When administered simultaneously they can be administered in a single composition, with the tetracycline and tetracycline-like compound(s) mixed in the blood-derived compositions. They blood-derived composition is preferably administered intravenously or intraperitoneally; the tetracycline and tetracycline-like compound is preferably administered orally. Multiple doses of each may be administered as needed. Precise dosage and regimen can be empirically determined.

[0156] The combination therapy may also include a known therapeutic treatment or regimen for a particular acute inflammatory disease, condition or disorder. Hence combinations of the blood-derived (or immune) compositions with tetracycline and/or tetracycline-like compounds are provided; combinations of the blood-derived (or immune) compositions with other therapeutic agents for treatment of a particular disorder, and combinations of the blood-derived (or immune) compositions with tetracycline and/or tetracycline-like compounds and with other therapeutic agents are provided. The component of combinations may be provided as separate compositions or may be provided as mixtures of two or more compositions. The tetracycline and tetracycline-like compounds are preferably administered orally and the blood-derived compositions are preferably administered by IV.

[0157] Kits containing the combinations are provided. The kits contain the components of the combinations, such as the blood-derived composition and tetracycline and/or tetracycline-like compounds, and optionally include instructions for administration to treat acute inflammatory response disorders. The reagents in the kits are packaged in standard pharmaceutical containers and packaging material. The kits may optionally contain additional components, such as syringes for administration of the compositions.

[0158] It is also shown herein that tetracycline and tetracycline-like compounds are effective for treatment of viral and bacterial infections, particularly, hemorrhagic fevers and infections with pathogenic *E. coli*. The tetracycline and tetracycline-like compounds may be administered with known treatments for hemorrhagic fevers. Combinations and kits containing the combinations of tetracycline and/or tetracycline-like compounds and such anti-hemorrhagic viral infections are also provided.

[0159] 1. Tetracycline-like compounds

[0160] Tetracycline-like compounds, which include thalidomide, aureomycin and sulfa drugs, and any other compound that exhibits tetracycline-like activity, either in the ability to induce expression of TNF and/or IL-1 receptors in treated individuals, which can be determined in model animals as in the Examples below, or by the ability to alter folic acid metabolism in bacteria. Such compounds can be identified empirically. Any compounds that can do either are suitable for use in the methods of treating acute inflammatory responses provided herein.

[0161] 2. Tetracycline compounds

[0162] a. Anti-inflammatory activity of tetracyclines

[0163] Tetracyclines are a well-known family of antibiotics that are active against a wide range of gram-positive and gram-negative bacteria. There are some indications in the art that tetracycline has anti-inflammatory activities, which are independent of its antibacterial activity (see, e.g., U.S. Pat. No. 5,773,430; U.S. Pat. No. 5,789,395; Shapira et al. (1996) *Infect. Immun.* 64:825-828; Kloppenburg et al. (1996) *Antimicrob. Agents. Chemother.* 40:934-940; Celerier et al. (1996) *Arch. Dermatol. Res.* 288:411-414; Milano et al. (1997) *Antimicrob. Agents. Chemother.* 41(1): 117-121; and U.S. Pat. No. 5,668,122). None, however, describe or suggest the use of tetracycline or tetracycline-like compounds for treatment of hemorrhagic fevers nor for production of blood-derived compositions for treatment of disorders, diseases and conditions characterized by or associated with an acute inflammatory response.

[0164] b. Exemplary tetracycline compounds

[0165] For purposes herein a tetracycline is any compound recognized by those of skill in the art to have the anti-inflammatory activities of a tetracycline and includes, all derivatives, including salts, esters and acids, analogs, prodrugs, modified forms thereof, and other compounds related to tetracycline as desribed above. The following are exemplary tetracycline compounds intended for use in the methods and compositions and combinations provided herein.

[0166] (1) Chlortetracycline

[0167] Chemically, chlortetracycline is 7-Chloro-4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide. Chemical synonyms of chlortetracycline include 7-chloro-tetracycline, Acronize, Aureocina, Aureomycin, Biomitsin, Biomycin and Chrysomykine. For purposes herein, the name "chlortetracycline" is used herein, although all such chemical synonyms are contemplated. Chemical synonyms of chlortetracycline hydrochloride include, but are not limited to, Aureociclina and Isphamycin.

[0168] Chlortetracycline can be prepared according to methods known in the art. For example, chlortetracycline can be isolated from the substrate of Streptomyces aureofaciens (Duggar, *Ann. N.Y. Acad. Sci.* 51, 177 (1948); U.S. Pat. No. 2,482,055 (1949 to Am Cyanamid); and Broschard et al., *Science* 109, 199 (1949)). Purification of chlortetracycline is described in Winterbottom, etal., U.S. Pat. No. 2,899,422 (1959 to Am. Cyanamid). Other processes for preparation of chlortetracycline is described in U.S. Pat. Nos. 2,987,449 and 3,050,446.

[0169] (2) Demeclocycline

[0170] Chemically, demeclocycline is 7-Chloro-4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-1,11-dioxo-2-naphthacenecarboxamide. Chemical synonyms of demeclocycline include 7-chloro-6-demethyltetracycline, demethylchlortetracycline (obsolete), RP 10192, Bioterciclin, Declomycin, Deganol, Ledermycin and Periciclina. For purposes herein, the name "demeclocycline" is used, although all such chemical synonyms are contemplated. Chemical synonyms of demeclocycline hydrochloride include, but are not limited to, Clortetrin, Demetraciclina, Detravis, Meciclin and Mexocine.

[0171] Demeclocycline can be prepared according to methods known in the art. For example, demeclocycline can be prepared according to the procedures described in McCormick et al., *J. Am. Chem. Soc.* 79, 4561 (1957); and U.S. Pat. No. 2,878,289 (1959 to Am. Cyanamid). Fermentation processes for demeclocycline preparation is described in U.S. Pat. Nos. 3,012,946, 3,019,172 and 3,050,446 (to Am. Cyanamid); Fr. pat. No. 1,344,645 (1963 to Merck & Co.); and Neidleman, U.S. Pat. No. 3,154,476 (1964 to Olin Mathieson). Demeclocycline hydrochloride is also available from Lederle Labs (Declomycin Tablets).

[0172] (3) Doxycycline

[0173] Chemically, doxycycline is 4-(Dimethylamino)-1,4,4a,5,5a,6,11,12a-octa-hydro-3,5,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide monohydrate. Other chemical synonyms of doxycycline include: α-6-deoxy-5-hydroxytetracycline monohydrate; α-6-deoxyoxytetracycline monohydrate; or 5-hydroxy-α-6-deoxytetracycline monohydrateGS-3065; Azudoxat; Doxitard; Doxy-Puren; Investin; Liviatin; Nordox; Spanor; Vibramycin; and Vibravenös. For consistency, only the name "doxycycline" is used herein, although the all such chemical synonyms are contemplated.

[0174] Chemical synonyms of "doxycycline hydrochloride" include doxycycline hyclate, Diocimex, Doryx, Doxatet, Doxigalumicina, Doxy-II (caps), Doxylar, Doxy-Tablinen, Doxytem, duradoxal, Ecodox, Granudoxy, Hydramycin, Liomycin, Mespafin, Midoxin, Nivocilin, Novadox, Retens, Roximycin, Samecin, Sigadoxin, Tanamicin, Tecacin, Tetradox, Vibradox, Vibramycin Hyclate, Vibra-Tabs and Zadorin.

[0175] Doxycycline can be prepared according to methods known in the art. For example, 6-doxytetracyclines can be prepared according to the procedures described in Wittenau et al., *J. Am. Chem. Soc.* 84:2645 (1962); Stephens et al. *J. Am. Chem. Soc.* 85, 2643 (1963); Blackwood et al., U.S. Pat. No. 3,200,149 (1965 to Pfizer). Preparation, separation and configuration of 6α- and 6β-epimers are described in Wittenau et al., *J. Am. Chem. Soc.* 84, 2645 (1962); Stephens et al., *ibid.* 85, 2643 (1963).

[0176] Doxycycline calcium is available from Pfizer (Vibramycin Calcium Oral Suspension Syrup). Doxycycline hyclate is available from Pfizer (Vibramycin Hyclate Capsules; Vibramycin Hyclate Intravenous; Vibra-Tabs Film Coated Tablets), from Warner Chilcott Professional Products (Doryx Coated Pellets), from Warner Chilcott (Doxycycline Hyclate Capsules) and from Mylan (Doxycycline Hyclate Capsules and Tablets). Doxycycline monohydrate is available from Pfizer (Vibramycin Monohydrate for Oral Suspension) and from Oclassen (Monodox Capsules).

[0177] (4) Methacycline

[0178] Chemically, methacycline is [4S-(4α,4aα,5α,5aα,12aα)]-4-Di-methylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,5,10,12,12a-pentahydroxy-6-methylene-1,11-dioxo-1-naphthacenecarboxamide. Chemical synonyms of methacycline include 6-methyleneoxytetracycline, 6-methylene-5-hydroxytetracycline, metacycline and Bialatan. For purposes herein, the name "methacycline" is used. It is understood that all chemical synonyms are contemplated. Chemical synonyms of methacycline hydrochloride include Andriamicina, Ciclobiotic, Germiciclin, Globociclina, Megamycine, Metadomus, Metilenbiotic, Londomycin, Optimycin, Physiomycine, Rindex and Rondomycin.

[0179] Demeclocycline can be prepared according to methods known in the art. For example, methacycline can be prepared from oxytetracycline (Blackwood et al., *J. Am. Chem. Soc.* 83 2773 (1961); 85, 3943 (1963); and Blackwood, U.S. Pat. No. 3,026,354 (1962 to Pfizer)).

[0180] (5) Minocycline

[0181] Chemically, minocycline is 4,7-Bis(dimethylamino)-1,4,4a,5,-5a,6,11,12a-octahydro-3,10,12,12a-tetrahydroxy-1,11-dioxo-2-naphthacenecarboxamide. Chemical synonyms of minocycline include 7-dimethylamino-6-demethyl-6-deoxytetracycline and Minocyn. For purposes herein, the name "minocycline" is used, but all such chemical synonyms are contemplated. Chemical synonyms of minocycline hydrochloride include Minocin, Klinomycin, Minomycin and Vectrin.

[0182] Minocycline can be prepared according to methods known in the art. For example, minocycline can be prepared according to the procedures described in Boothe, Petisi, U.S. Pat. Nos. 3,148,212 and 3,226,436 (1964 and 1965 to Am. Cyanamid). Synthesis of minocycline is described in Martell, Boothe, *J. Med. Chem.* 10, 44 (1967); Church etal., *J. Org. Chem.* 36, 723 (1971); and Bernardi et al., *Farmaco Ed. Sci.* 30, 736 (1975). Minocycline hydrochloride is available from Medicis (Dynacin Capsules), from Lederle Labs (Minocin Intravenous; Minocin Oral Suspension; and Minocin Pellet-Filled Capsules) and from Warner Chilcott Professional Products (Vectrin Capsules).

[0183] (6) Oxytetracycline

[0184] Chemically, oxytetracycline is 4-(Dimethylamino)-1,4,4a,5,-5a,6,11,12a-octahydro-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide. Chemical synonyms of oxytetracycline include: glomycin; terr-fungine; riomitsin; hydroxytetracycline; Berkmycen; Biostat; Engemycin; Oxacycline; Oxatets; Oxydon; Oxy-Dumocyclin; Oxymycin; Oxypan; Oxytetracid; Ryomycin; Stevacin; Terraject; Terramycin; Tetramel; Tetran; Vendarcin; and Vendracin. For purposes herein, the name "oxytetracycline" is used, although all such chemical synonyms are contemplated. Chemical synonyms of oxytetracycline dihydrate include Abbocin, Clinimycin and Imperacin. Chemical synonyms of oxytetracycline hydrochloride dihydrate include Alamycin, Aquacycline, Arcospectron, Bio-Mycin, Duphacycline, Geomycin, Gynamousse, Macocyn, Macodyn, Occrycetin, Oxlopar, Oxybiocycline, Oxybiotic, Oxycycline, Oxyject, Oxylag, Stecsolin, Tetra-Tablinen and Toxinal.

[0185] Oxytetracycline can be prepared according to methods known in the art. For example, oxytetracycline can be isolated from the elaboration products of the antinomycete, *Streptomyces rimosus*, grown on a suitable medium (Finlay et al., *Science* 111, 85 (1950); Regna, Solomons, *Ann. N.Y. Acad. Sci.* 53, 221 (1950); Regna et al., *J. Am. Chem. Soc.* 73, 4211 (1951)), from *Streptomyces rimosus* (Sobin etal., U.S. Pat. No. 2,516,080 (1950 to Pfizer)), from *S. xanthophaeus* (Brockmann, Musso, *Naturwiss.* 41, 451 (1954); Brockmann et al., Ger. pat. 913,687 (1954 to Bayer), *C.A.* 53, 4662h (1959)). Total synthesis of the dl-form of oxytetracycline is described in H. Muxfeldt et al., *ibid.* 101, 689 (1979). Oxytetracycline hydrochloride is available from Pfizer (Terra-Cortril Ophthalmic Suspension; Terramycin with Polymyxin B Sulfate Ophthalmic Ointment; and Urobiotic-250 Capsules).

[0186] (7) Tetracycline

[0187] Chemically, tetracycline is 4-dimethylamino)-1,4,4a,5,5a,6-11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide. Chemical synonyms of tetracycline include deschlorobiomycin; tsiklomitsin; Abricycline; Achromycin; Agromicina; Ambramicina; Ambramycin; Bio-Tetra; Bristaciclina; Cefracycline suspension; Criseociclina; Cyclocmycin; Democracin; Hostacyclin; Omegamycin; Panmycin; Polycycline; Purocyclina; Sanclomycine; Steclin; Tetrabon; Tetracyn; Tetradecin. For purposes herein, the name "tetracycline", although the all such chemical synonyms are contemplated.

[0188] Chemical synonyms (i.e. equivalents or generics) tetracycline hydrochloride, include Achro, Achromycin V, Ala Tet, Ambracyn, Artomycin, Cefracycline tablets, Cyclopar, Diacycline, Dumocyclin, Helvecyclin, Imex, Mephacyclin, Partrex, Quadracycline, Quatrex, Remicyclin, Ricycline, Ro-cycline, Stilciclina, Subamycin, Supramycin, Sustamycin, Tefilin, Teline, Telotrex, Tetrabakat, Tetrabid, Tetrablet, Tetrachel, Tetracompren, Tetra-D, Tetrakap, Tetralution, Tetramavan, Tetramycin, Tetrosol, Tetra-Wedel, Topicycline, Totomycin, Triphacyclin, Unicin, Unimycin and Vetquamycin-324. Chemical synonyms of tetracycline phosphate complex include Panmycin Phosphate, Sumycin, Tetradecin Novum, Tetrex and Upcyclin.

[0189] In addition to its ubiquitous commercial availability, tetracycline can be prepared according to methods known in the art. For example, tetracycline can be produced from Streptomyces spp. (Boothe et al. *J. Am. Chem. Soc.* 75, 4621 (1953); Conover et al., *ibid.* 4622; and Conover, U.S. Pat. No. 2,699,054 (1955)), from *Streptomyces viridifaciens* (Gourevitch, et al., U.S. U.S. Pat. Nos. 2,712,517; 2,886,595 (1955, 1959 to Bristol Labs)), from *S. aureofaciens* (U.S. Pat. Nos. 3,005,023; 3,019,173). Purification of tetracycline is described, for example, in U.S. Pat. No. 3,301,899. Preparation of tetracycline phosphate complex is described in Seiger, Weidenheimer, U.S. Pat. No. 3,053,892 (1962 to Am. Cyanamid). Total synthesis of tetracyclines is described in Boothe et al., *J. Am. Chem. Soc.* 81, 1006 (1959); Conover et al., ibid. 84, 3222 (1962). Tetracycline hydrochloride is available from Lederle Labs (Achromycin V Capsules), from Procter & Gamble Pharmaceutical (Helidac Therapy), from Lederle Standard (Tetracycline HCl Capsules) and from Mylan (Tetracycline Hydrochloride Capsules). Soluble tetracycline is preferred.

[0190] (8) Other Chemically-Modified Tetracyclines

[0191] Other tetracyclines include, but are not limited to, dedimethylaminotetracyclines, which include 4-dedimethylaminotetracycline, 4-dedimethylamino-5-oxytetracycline, 4-dedimethylamino-7-chlortetracycline, 4-hydroxy-4-dedimethyl-aminotetracycline, 5a, 6-anhydro-4hydroxy-4-dedimethylaminotetracycline, 6α-deoxy-5-hydroxy-4-dedimethylaminotetracycline, 6-demethyl-6-deoxy-4-dedimethylaminotetracycline, 4-dedimethylamino-12a-deoxytetracycline, 4-dedimethylamino-11-hydroxy-12a-deoxytetracycline, 12a-deoxy-4-deoxy-4-dedimethylaminotetracycline, 6α-deoxy-5-hydroxy-4-dedimethylaminodoxycycline, 12a,4a-anhydro-4-dedimethylaminotetracycline and minocycline-CMT i.e., 7-dimethylamino-6-demethyl-6-deoxy-4-dedimethylaminotetracycline. Further examples of chemically-modified tetracyclines contemplated for use herein, include but are not limited to, 6a-benzylthiomethylenetetracycline, the 2-nitrilo analogs of tetracycline (tetracyclinonitrile), the mono-N-alkylated amide of tetracycline, 6-fluoro-6-demethyltetracycline, 11a-chlortetracycline, tetracycline pyrazole and 12a-deoxytetracycline and its derivatives (see, e.g., U.S. Pat. No. 5,532,227).

[0192] Other chemically modified tetracyclines (CMT's) include, but are not limited to for example, 4-de(dimethylamino)tetracycline (CMT-1), tetracyclinonitrile (CMT-2), 6-demethyl-6-deoxy-4-de(dimethylamino)tetracycline (CMT-3), 7-chloro-4-de(dimethylamino)tetracycline (CMT-4), tetracycline pyrazole (CMT-5), 4-hydroxy-4-de(dimethylamino)tetracycline (CMT-6), 4-de(dimethylamino)-12.alpha.-deoxytetracycline (CMT-7), 6-deoxy-5.alpha.-hydroxy-4-de(dimethylamino)tetracycline (CMT-8), 4-de(dimethylamino)-12.alpha.-deoxyanhydrotetracycline (CMT-9) and 4-de(dimethylamino)minocycline (CMT-10) (see, e.g., U.S. Pat. No. 5,773,430). Further examples of tetracyclines modified for reduced antimicrobial activity include the 4-epimers of oxytetracycline and chlortetracycline (epi-oxytetracycline and epichlortetracycline).

[0193] Also contemplated and included are 4-dedimethylaminotetracyclines and the corresponding 5a,6-anhydro derivatives having an oxo, hydroxy, substituted imino, amino or substituted amino group other than dimethylamino at the C.multidot.4-position useful as antimicrobial agents. Examples of such 4-dedimethylaminotetracyclines derivatives include 5-Oxytetracycline, 7-Chlortetracycline, 6-Deoxy-5-oxytetracycline, 6-Deoxytetracycline, 6-Deoxy-6-demethyltetracycline, 7-Bromotetracycline, 6-Demethyl-7-chlortetracycline, 6-Demethyltetracycline, 6-Methylenetetracycline, 11a-Chloro-6-methylenetetracycline, 6-Methylene-5-oxytetracycline and 11a-Chloro-6-methylene-5-oxytetracycline (see, e.g., U.S. Pat. No. 4,066,694).

[0194] Aqueous solutions of chlortetracycline or salts thereof, a pharmaceutically acceptable calcium compound and 2-pyrrolidone as a co-solvent, where the solution has a pH of 8 to 10 is used as an injectable composition combining low viscosity, high potency, good clarity and good stability (see, U.S. Pat. No. 4,081,527).

[0195] Further, the tetracycline compounds and formulations that can be used herein include those compounds or formulations described in the following U.S. Pat. Nos. or

those compounds or formulations that can be prepared according to the processes described in the following U.S. Pat. Nos.:

[0196] 5,827,840 (Chemically-modified tetracyclines); 5,789,395 (Method of using tetracycline compounds for inhibition of endogenous nitric oxide production); 5,773,430 (Serine proteinase inhibitory activity by hydrophobic tetracycline); 5,770,588 (Non-antibacterial tetracycline compositions); 5,668,122 (Method to treat cancer with tetracyclines); 5,538,954 (Salts of tetracyclines); 5,532,227 (Tetracyclines including non-antimicrobial chemically-modified tetracyclines); 5,523,297 Non-antimicrobial tetracyclines); RE34,656 (Use of tetracycline to enhance bone protein synthesis and/or treatment of bone deficiency); 5,321,017 (Composition containing fluriprofen and effectively non-antibacterial tetracycline to reduce bone loss); 5,308,839 (Composition containing non-steroidal anti-inflammatory agent tenidap and effectively non-antibacterial tetracycline); 5,277,916 (Tetracycline dosage form); 5,258,372 (Tetracycline activity enhancement using doxycycline or sancycline); 5,250,442 (Method of treating rheumatoid arthritis using tetracycline); 5,223,248 (Non-antibacterial tetracycline compositions possessing antiplaque properties); 5,021,407 (Tetracycline activity enhancement); 4,935,412 (Non-antibacterial tetracycline compositions possessing anti-collagenolytic properties and methods of preparing and using same); 4,935,422 (Non-antibacterial tetracycline compositions possessing anti-collagenolytic properties and methods of preparing and using same); 4,925,833 (Use of tetracycline to enhance bone protein synthesis and/or treatment of osteoporosis); 4,837,030 (Novel controlled release formulations of tetracycline compounds); 4,704,383 (Non-antibacterial tetracycline compositions possessing anti-collagenolytic properties and methods of preparing and using same); 4,666,897 (Inhibition of mammalian collagenolytic enzymes by tetracyclines); 4,418,060 (Therapeutically active complexes of tetracyclines); 4,376,118 (Stable non-aqueous solution of tetracycline salt); 4,081,528 (Tetracycline compositions); 4,066,694 (4-Hydroxy-4-dedimethylamino-tetracyclines); 4,060,605 (Water-soluble derivative of 6-deoxy-tetracyclines); 3,993,694 (Tetracycline derivatives and process for preparing them); 3,983,173 (2-Carboxamido-substituted tetracyclines and process for their manufacture); 3,962,330 (Process for the preparation of 6-demethyl-6-deoxy-6-methylene-tetracyclines); 3,947,517 (Stereoselective introduction of tetracyclines hydroxyl group at 12(a) position in synthesis of tetracyclines); 5,387,703 (Process and intermediate for the purification of oxytetracycline); 5,075,295 (Novel oxytetracycline compositions); 4,829,057 (Oxytetracycline capsules with increased stability and methods of producing the same); 4,584,135 (Process for the preparation of an oxytetracycline-calcium silicate complex salt from fermentation broth); 4,399,127 (Injectable oxytetracycline compositions); 4,386,083 (Injectable oxytetracycline compositions); 4,259,331 (Oxytetracycline compositions); 4,020,162 (Oxytetracycline solution for parenteral, peroral and local administration and processes for the production thereof); 4,018,889 (Oxytetracycline compositions); 3,962,435 (Combination of oxytetracycline and 2,4-diamino-5-(3-alkoxy-4,5-methylenedioxybenzyl)pyrimidine); 3,962,131 (Rhodium containing catalyst and use thereof in preparation of 6-deoxy-5-oxytetracycline); 3,957,972 (Stable solutions of oxytetracycline suitable for parenteral and peroral administration and process of preparation); 5,258,372 (Tetracycline activity enhancement using doxycycline or sancycline); 4,086,332 (Doxycycline compositions); 4,061,676 (Recovery of doxycycline and products thereof); 3,957,980 (Doxycycline parenteral compositions); 3,932,490 (Doxycycline aceturate); 5,413,777 (Pulsatile once-a-day delivery systems for minocycline); 5,348,748 (Pulsatile once-a-day delivery systems for minocycline); 5,300,304 (Pulsatile once-a-day delivery systems for minocycline); 5,262,173 (Pulsatile once-a-day delivery systems for minocycline); and 4,701,320 (Composition stably containing minocycline for treating periodontal diseases).

[0197] Hence tetracycline compounds are well known to those of skill in the art; and tetracycline-like compounds can be readily identified.

[0198] C. Hemorrhagic Viruses and the Immune Response

[0199] The immune response to hemorrhagic viral infection appears to follow the a scheme that includes: viral activation of macrophages, T and B lymphocytes; production of mediators by mononuclear cells, including cytokines such as, interleukin (IL)-1 and IL-2, interferon (IFN), and/or tumor necrosis factor (TNF); changes of the proliferative activity of the cells; alterations of lymphocyte subpopulations (CD4 and CD8); and propagation of virus in immunocompetent cells.

[0200] A decrease of lymphocyte proliferative activity in response to mitogen stimulation, a decrease of the number of T and B lymphocytes, and an inversion of CD4\CD8 lymphocyte ratios (Fisher-Hoch etal. (1987) *J. Infect. Dis.*, 155:465-474; Vallejos et al. (1985) *Medicina* (Buenos-Aries), 45:407; Enria et al. (1986) *Med. Microbiol. Immunol*, 175:173-176) have been demonstrated in arenaviral hemorrhagic fevers.

[0201] Clinical observations and experimental study of these fevers has demonstrated a marked production of the inflammatory cytokines, such as TNF, IL-1, IFN, during these diseases. Pronounced production of serum IFN was seen during experimental infection of guinea pigs and monkeys with Marburg and Ebola viruses with lethal outcome (Ognatyev et al., *Voprosy Virusologii*, 39:13-17 (1994); Ignatyev et al., *Voprosy Virusologii*, 40:109-113 (1995); Ignatyev et al., *J. Biotechnol*, 44:111-118 (1996)). The infection of human macrophages with Marburg virus leads to increased release of TNF-α, which is one of several cytokines typically secreted by macrophages (Feldmann et al., *J. Virol.*, 70:2208-2214 (1996)). Infection of monkeys with Ebola virus was also accompanied by increased serum TNF-α levels (Ignatyev, *Curr. Top. Microbiol. Immunol.*, 235:205-217 (1999)).

[0202] Increased levels of TNFα and IFN-α in patients with Argentine hemorrhagic fever correlate with the severity of disease; whereas IL-1β levels in patients do not differ from those in normal controls (see, Heller et al., *J. Infect. Dis.*, 166:1203 (1992)). Increased production of nitric oxide (NO) in patients with hemorrhagic fever with renal syndrome has been reported (Linderholm et al., *Infection*, 24:337-340 (1996)).

[0203] Similarly high concentrations of IL-1 and TNF during the development of the human septic shock are known to contribute to lethal outcome (see, Calandra et al.,

J. Infectious Diseases, 161:982-987 (1990); Cannon et al., *J. Infectious Diseases*, 161 :79-84 (1990)).

[0204] Defective humoral responses and extensive intravascular apoptosis are associated with fatal outcome in ebola virus-infected patients (Baize et al., *Nature Medicine*, 5(4):423-426 (1999)). In survivors, early and increasing levels of IgG, directly against mainly against the nucleoprotein and the 40-kDa viral protein, were followed by clearance of circulating viral antigen and activation of cytotoxic T cells. In contrast, fatal infection was characterized by impaired humoral responses, with absent specific IgG and barely detectable IgM.

[0205] The compositions and method provided herein provide a means to treat infections with hemorrhagic viruses. In particular, the blood-derived compositions, which can be readily produced by contacting blood from a donor in vitro or in vivo with a compound such as as, a tetracycline or tetracycline-like compound, and then harvesting, preferably serum or plasma, which can be infused into the mammal with the infection, is effective for treatment. The response in the donor blood or fraction thereof can be observed as quickly as six hours after administration of the tetracycline and tetracycline-like compound or contacting with the blood. The infected mammal can also be treated with a tetracycline and tetracycline-like compounds prior to administration of the blood-derived composition, simultaneously and/or subsequently. Additional anti-hemorrhagic viral treatments and agents may also be administered.

[0206] D. Pharmaceutical Compositions, Formulation and Modes of Administration Thereof

[0207] Blood-derived compositions for administration, preferably for systemic administration, for treatment of acute inflammatory responses are provided. These are preferably provided in a form for systemic, such as intraperitoneal or intravenous administration. They may be concentrated or diluted by standard methods; preferably they are not subjected to freeze-drying.

[0208] Combinations of the blood-derived compositions with tetracycline and/or tetracycline-like compounds are also provided. These combinations may be packaged as kits and are intended for treatment of the acute inflammatory responses.

[0209] Also provided for treatment of the viral hemorrhagic diseases and also bacterial infections, such as *E. coli*, are tetracycline and tetracycline-like compounds, and also combinations of a composition containing one or more tetracycline compoundis) and a composition containing an anti-viral-hemorrhagic agent, preferably in a pharmaceutically acceptable carrier or excipient. The tetracycline compound(s) and anti-viral-hemorrhagic agent are packaged as separate compositions for administration together or sequentially or intermittently. Alternatively, they can be contained in a single composition for administration as a single composition. The combinations can be packaged as kits.

[0210] In a preferred embodiment, a composition suitable for oral delivery, includes one or more tetracycline compounds and an anti-viral-hemorrhagic agent, and a pharmaceutically acceptable carrier or excipient in tablet, capsule, or other single unit dosage form is provided.

[0211] Any tetracycline and tetracycline-like compound(s), including those described herein, when used alone or in combination with other compounds, that can alleviate, reduce, ameliorate, prevent, or place or maintain in a state of remission of clinical symptoms or diagnostic markers associated with acute inflammatory responses, such as viral hemorrhagic diseases or disorders, particularly those viral hemorrhagic diseases or disorders caused by infection of a Bunyaviridae, a Filoviridae, a Flaviviridae, or an Arenaviridae virus, can be used in the present combinations.

[0212] Suitable anti-viral hemorrhagic agents are described in the following section.

[0213] 1. Anti-viral hemorrhagic agents

[0214] The tetracycline and tetracycline-like compounds and the blood-derived compositions provided herein can be administered alone or in combination with other agents, such as IL-1 inhibitors and/or TNF inhibitors, appropriate vaccines and other drugs for treatment of acute inflammatory diseases and disorders.

[0215] a. Interleukin-1 (IL-1) inhibitors

[0216] Any IL-1 inhibitor that prevents or decreases production, post-translational modification(s), maturation, or release of IL-1, or any substances that interfere with or decrease the efficacy of the interaction between IL-1 and IL-1 receptor is contemplated for use in combination with the tetracycline and tetracycline-like compounds and/or the blood-derived compositions. Preferably, the IL-1 inhibitor is an anti-IL-1 antibody, an anti-IL-1 receptor antibody, an IL-1 receptor antagonist, an IL-1 production inhibitor, an IL-1 receptor production inhibitor and an IL-1 releasing inhibitor.

[0217] Monoclonal antibodies, particularly humanized antibodies are preferrred. Anti-IL-1 antibodies are known (see, e.g., U.S. Pat. Nos. 4,772,685 and 4,994,553). Anti-IL-1 receptor antibodies are also known (see, e.g., Chen et al., *Cancer Res.*, 58(16): 3668-76 (1998); Clark et al., *J. Interferon Cytokine Res.*, 16(12): 1079-88 (1996); Zerek-Melen et al., *Eur. J. Endocrinol.*, 131(5): 531-4 (1994); McIntyreet al. (1991) *J. Exp. Med.*, 173(4):931-9; Benjamin et al. (1990) *Prog. Clin. Biol. Res.*, 349:355-6) are used.

[0218] An IL-1 receptor antagonist can be an IL-1 receptor antagonist (IL-1Ra; see, e.g., SEQ ID No. 5; see, also U.S. Pat. Nos. 5,863,769, 5,837,495, 5,739,282, 5,508,262, 5,455,330, 5,334,380, Bendele et al., *Arthritis Rheum.*, 42(3):498-506 (1999); Kuster et al., *Lancet*, 352(9136):1271-7 (1998); Bendele et al., *J. Lab. Clin. Med.*, 125(4): 493-500 (1995); and Wetzler et al., *Blood*, 84(9):3142-7 (1994)), an IL-1 receptor intracellular ligand protein, a Type II IL-1 receptor, a soluble IL-1 receptor, a non-functional mutein of IL-1, a non-functional mutein of IL-1 receptor or a small molecule antagonist.

[0219] IL-1 receptor intracellular ligand proteins (see, e.g., SEQ ID Nos. 6, 7, 8 and 9; see also U.S. Pat. No. 5,817,476), such as type 11 IL-1 receptor (see, e.g., SEQ ID No. 4; see, also U.S. Pat. Nos. 5,464,937 and 5,350,683) or soluble IL-1 receptors (see, e.g., U.S. Pat. Nos. 5,767,064, RE35,450, 5,492,888, 5,488,032, 5,319,071 and 5,180,812) are contemplated. Soluble receptors contain residues 1-312, 1-314, 1-315, 1-316, 1-317, 1-318 and 1-319 of the full-length receptor for which sequence is set forth in SEQ ID No. 3 or 4). Non-functional muteins of IL-1 (see, e.g., U.S. Pat. No. 5,286,847) can be used (e.g., in which the Arg

residue at position 127 of the precursor IL-1β protein sequence (see, SEQ ID No. 2) is replaced with gly). The small molecule IL-1 receptor antagonist can be a histamine antagonist (see, e.g., U.S. Pat. No. 5,658,581), an aryl-or heteroaryl-1-alkyl-pyrrole-2-carboxylic acid compound (see, e.g., U.S. Pat. Nos. 5,039,695 and 5,041,554) or a 5-lipoxygenase pathway inhibitor (U.S. Pat. No. 4,794,114).

[0220] The IL-1 inhibitor can be an IL-1 production inhibitor, such as an antisense oligonucleotide (see, e.g., Yahata et al., *Antisense Nucleic Acid Drug Dev.*, 6(1):55-61 (1996); Fujiwara et al., *Cancer Res.*, 52(18): 4954-9 (1992); see, also SEQ ID. No. 10, which sets forth an exemplary anti-sense oligonucleotide specific for IL-1,6; and Maier et al., *Science,* 249:1570-4 (1990); SEQ ID No. 11, which sets forth an exemplary antisense oligonucleotide specific for IL-1 α) can be used.

[0221] The IL-1 production inhibitor can be a small molecule inhibitor, such as 5-hydroxy and 5-methoxy 2-aminopyrimidine (see, e.g., U.S. Pat. No. 5,071,852), 3-substituted-2-oxindole-1-carboxamide (see, e.g., U.S. Pat. Nos. 4,861,794 and 5,192,790), 4,5-diaryl-2(substituted)imidazole (see, e.g., U.S. Pat. No. 4,780,470) and 2-2'-[1,3-propan-2-onediyl-bis(thio)]bis-1-H-imidazole (see, e.g., U.S. Pat. No. 4,778,806).

[0222] The IL-1 inhibitor can be an IL-1 receptor production inhibitor, such as an antisense oligonucleotide (see, e.g., SEQ ID No. 12, which provides an antisense oligonucleotide designated ISIS 8807; see, also Miraglia et al., *Int. J. Immunopharmacol.,* 18(4):227-40 (1996); the oligonucleotide set forth in SEQ ID No. 13; and Burch et al., *J. Clin. Invest.,* 88(4):1190-6 (1991)) can be used.

[0223] The IL-1 inhibitor can be an IL-1 releasing inhibitor, such as an IL-1 converting enzyme inhibitor e.g., N-substituted glutamic acid derivative (see, U.S. Pat. No. 5,744,451), y-pyrone-3-acetic acid (U.S. Pat. No. 5,411, 985), probucol (U.S. Pat. No. 4,975,467), disulfiram, tetrakis [3-(2,6-di-tert-butyl-4-hydroxyphenyl)propionyloxy methyl]methane or 2,4-di-isobutyl-6-(N,N-dimethylaminomethyl)-phenol (U.S. Pat. No. 5,034,412), a peptide based interleukin-1 beta converting enzyme (ICE) inhibitor (Okamoto et al., *Chem. Pharm. Bull.* (Tokyo) 47(1):11-21 (1997)), a pyridazinodiazepine (Dolle et al., *J. Med. Chem.,* 40(13):1941-6 (1997)), SDZ 224-015 (Elford et al., *Br. J. Pharmacol.,* 115(4):601-6 (1995)), an aspartate-based inhibitor (Mashima et al., *Biochem. Biophys. Res. Commun.,* 209(3):905-15 (1995)), an aspartyl alpha-((1-phenyl-3-(trifluoromethyl)-pyrazol-5-yl)oxy)methyl ketone (Dolle et al., *J. Med. Chem.,* 37(23):3863-6 (1994)), L-741,494 (Salvatore et al., *J. Nat. Prod.,* 57(6):755-60 (1994); see U.S. Pat. No. 5,843,904), TX (see U.S. Pat. No. 6,020,477), CPP-32 and CMH-1 (Margolin et al., *J. Biol. Chem.,* 272(11):7223-8 (1997)), a peptide inhibitor of ICE, YVAD-CHO (de Bilbao et al., *Neuroreport,* 7(18):3051-4 (1996)), benzyloxycarbonyl-valinylaianylaspartylfluoromethyl ketone (Cain et al., *J. Biochem.,* 314(Pt 1):27-32 (1996)) bocaspartyl (benzyl) chloromethylketone (BACMK) (Estrov et al., *Blood,* 86(12):4594-602 (1995)) and L-709,049 (Fletcher et al., *J. Interferon Cytokine Res.,* 5(3):243-8 (1995)).

[0224] Other IL-1 inhibitors may also be used (see, e.g., U.S. Pat. No. 5,804,599 (Interleukin-1 production inhibiting compound), U.S. Pat. No. 5,453,490 (Recombinant human interleukin-1 inhibitors), U.S. Pat. No. 5,334,380 (Anti-endotoxin, interleukin-1 receptor antagonist), U.S. Pat. No. 5,075,222 (Interleukin-1 inhibitors), U.S. Pat. No. 5,034,412 (Interleukin-1 release inhibitors), U.S. Pat. No. 5,011,857 (Interleukin-1 release inhibitors), U.S. Pat. No. 4,975,467 (Interleukin-1 release inhibitors), U.S. Pat. No. 4,870,101 (Interleukin-1 release inhibitors) and Ray et al., *Cell,* 69(4):597-604 (1992) (Cowpox virus encoded interleukin-1 beta converting enzyme inhibitor).

[0225] b. Tumor necrosis factor (TNF) inhibitors

[0226] TNF inhibitors may also be used. These may be used in place of or in addition to IL-1 inhibitors. Any inhibitor of TNF activity is contemplated for use herein. Among the preferred inhibitors are anti-TNF antibodies, anti-TNF receptor antibodies, TNF receptor antagonists, TNF production inhibitors, TNF receptor production inhibitors and a TNF releasing inhibitors.

[0227] The anti-TNF antibody or the anti-TNF receptor antibody can be a monoclonal antibody, which is preferably, humanized. Such antibodies are known (e.g., the anti-TNF antibodies Mabp55r and Mabp75r (Tanaka et al., *Neurol. Med. Chir. (Tokyo),* 38(12):812-818 (1998)), 3B10 and h3B10-9 (Nagahira et al., *J. Immunol. Methods.,* 222(1-2):83-92 (1999)), MAK 195F (Holler et al., *Blood.,* 86(3):890-0 (1995)), CA2 (Centocor, Inc., Malvern, Pa.; Elliott et al., *Lancet,* 344:1125-1127 (1994); Cope et al., *J. Clin. Invest.,* 94:749-760 (1994)) and CDP571 (Rankin et al., *Br. J. Rheumatol.,* 34(4):334-342 (1995); U.S. Pat. Nos. 5,741,488, 5,698,195, 5,654,407, 5,626,321, 5,656,272, 5,436,154, 5,360,716, 5,231,024 and 5,795,967; and Cargile et al., *Am. J. Vet. Res.,* 56(11):1451-9 (1995)).

[0228] The TNF receptor antagonist can be a purified soluble TNF receptor, a non-functional mutein of TNF receptor, a non-functional mutein of TNF and a small molecule antagonist. Non-functional muteins of TNF receptor are known (see, e.g., U.S. Pat. Nos. 5,863,786, 5,773, 582, 5,606,023, 5,597,899, 5,519,119, 5,486,463, 5,422,104, 5,247,070 and 5,028,420). Small molecule antagonists, such as a mercapto alkyl peptidyl compound (see, e.g., U.S. Pat. No. 5,872,146), an arylsulfonyl hydroxamic acid derivative (U.S. Pat. No. 5,861,510), a salt of an alkaline-earth metal (U.S. Pat. No. 5,851,556), a pentoxifylline (U.S. Pat. No. 5,763,446), a hydroxamic acid compound (U.S. Pat. No. 5,703,092), a retinoic acid (U.S. Pat. No. 5,658,949), a histamine antagonist (U.S. Pat. No. 5,658,581), a leflunomide (U.S. Pat. No. 5,547,970), a 1-Alkoxy-2-(alkoxy- or cycloalkoxy-)-4-(cyclothioalkyl- or cyclothioalkenyl-) benzene (U.S. Pat. No. 5,541,219), a vinigrol (U.S. Pat. No. 5,306,732), a cyclohexene-ylidene derivative (U.S. Pat. No. 5,605,923), a quinazoline compound (U.S. Pat. No. 5,646, 154) and BN 50739 (Rabinovici et al., *J. Pharmacol. Exp. Ther.,* 255(1):256-63 (1990)) are also contemplated for use herein in combination with the tetracycline and tetracycline-like compounds and/or blood-derived compositions.

[0229] The TNF receptor antagonist can be a TNF receptor death domain ligand protein, a tumor necrosis factor binding protein (TNF-BP), a TNF receptor-IgG heavy chain chimeric protein (Peppel et al., *J. Exp. Med.,* 174(6):1483-9 (1991)), a bacterial lipopolysaccharide binding peptide derived from CAP37 protein (U.S. Pat. No. 5,877,151) and a Myxoma virus T2 protein (Schreiber et al., *J. Biol. Chem.,* 271(23):13333-41 (1996)). Exemplary TNF receptor death domain ligand proteins include those described in U.S. Pat.

Nos. 5,849,501, 5,847,099, 5,843,675, 5,852,173 and 5,712, 381 are used (see, also SEQ ID Nos. 18, 19, 20 and 21). Also, the TNF-BPs described in U.S. Pat. No. 5,811,261, which describes TBP-1 a 180 amino acid protein isolated from human urine, U.S. Pat. Nos. 5,808,029, 5,776,895, 5,750,503, which describe chimeric TNF-BPs containing the soluble portion of the P55 TNF receptor and all but the first domain of the constant region of IgG1 or IgG3 heavy chains, and the TNF-BPs described in Colagiovanni et al., *Immunopharmacol. Immunotoxicol.,* 18(3):397-419 (1996) and Olsson et al., *Biotherapy.,* 3(2):159-65 (1991), which describes a 50 kD protein isolated from human urine, can be used.

[0230] The TNF inhibitor can be an TNF production inhibitor, such as an antisense oligonucleotide (see, e.g., SEQ ID No. 22; see, also U.S. Pat. No. 5,705,389). Other TNF production inhibitors are known (see, e.g., U.S. Pat. No. 5,776,947 (quinoline-3-carboxamide compounds), U.S. Pat. No. 5,691,382 (matrix metaloproteinase inhibitors), U.S. Pat. No. 5,648,359, U.S. Pat. No. 5,616,490 (ribozymes targeted to TNFα RNA), U.S. Pat. Nos. 5,304,634, 5,420, 154 and 5,547,979 (derivatives of 2-pyrrolidinones)).

[0231] TNF receptor production inhibitor include antisense oligonucleotides. The TNF inhibitor can be a TNF releasing inhibitor (see, e.g., U.S. Pat. No. 5,869,511 (isoxazoline compounds), U.S. Pat. No. 5,563,143 (catechol diether compounds), and U.S. Pat. No. 5,629,285 (peptidyl derivatives having active groups capable of inhibiting TACE such as, hydroxamates, thiols, phosphoryls and carboxyls)

[0232] Other TNF inhibitors are contemplated (see, e.g., U.S. Pat. No. 5,886,010 (TNFα inhibitors), U.S. Pat. No. 5,753,628 (peptide inhibitors of TNF containing predominantly D-amino acids), U.S. Pat. No. 5,695,953 (DNA that encodes a tumor necrosis factor inhibitory protein), U.S. Pat. No. 5,672,347 (tumor necrosis factor antagonists), U.S. Pat. No. 5,582,998 (monoclonal antibodies against human TNF-BP I), U.S. Pat. No. 5,478,925 (multimers of the soluble forms of TNF receptors), U.S. Pat. No. 5,464,938 (isolated viral protein TNF antagonists), U.S. Pat. No. 5,359,039 (isolated poxvirus A53R-equivalent tumor necrosis factor antagonists), U.S. Pat. No. 5,136,021 (TNF-inhibitory protein), U.S. Pat. No. 5,118,500 (xanthine derivatives), U.S. Pat. No. 5,519,000 (peptides that include 4-25 amino acids and bind to tumor necrosis factor-α) and U.S. Pat. No. 5,641,751.

[0233] C. Anti-viral vaccine, antibody and virally-activated immune cells and serum

[0234] For treatment of viral infections, particularly hemorrhagic fever infections, the tetracycline or tetracycline-like compounds and/or blood-derived composition may be administered in combination with an anti-viral vaccine, antibody and/or virally activated immune cells or serum.

[0235] Any anti-viral vaccines, anti-viral antibodies, viral-activated immune cells and viral-activated immune serums, when used alone or in combination with other compounds, that can alleviate, reduce, ameliorate, prevent, or place or maintain in a state of remission of clinical symptoms or diagnostic markers associated with viral hemorrhagic diseases or disorders, particularly those viral hemorrhagic diseases or disorders caused by infection of a Bunyaviridae, a Filoviridae, a Flaviviridae, or an Arenaviridae virus, can be used in the present combinations and in the methods of treatment in combination with administration of a tetracycline compound. Exemplary anti-viral treatments are agents include but are not limited to the following.

[0236] (1) Anti-viral vaccine

[0237] Anti-viral vaccines can be prepared according to the methods known in the art (see Current Protocols in Immunology (Ed. Coligan et al.) John Wiley & Sons, Inc., 1997). Any types of vaccines, including attenuated viruses, protein or peptide vaccines or nucleotide vaccines can be used.

[0238] (a) Anti-Bunyaviridae Vaccine

[0239] An anti-Bunyaviridae vaccine, preferably, an anti-Hantaan virus vaccine (see, e.g., U.S. Pat. No. 5,298,423 (nucleotide sequences coding for Hantaan virus nucleocapsid protein and glycoproteins G1 and G2), U.S. Pat. No. 5,183,658 (the purified and inactivated Hantaan virus ROK84/105), Chu, et al., *J. Virol.,* 69(10):6417-23 (1995) (a vaccinia virus-vectored vaccine expressing the M and the S segments of Hantaan (HTN) virus)) can be used.

[0240] (b) Anti-Filoviridae Vaccine

[0241] An anti-Filoviridae vaccine, such as an anti-ebola virus vaccine is used (e.g., the vaccines described in Chupurnov, et al., *Vopr. Virusol.,* 40(6):257-60 (1995) (inactivated viral agents (Nonlethal strain of the virus)), Lupton, et al., *Lancet,* 2(8207):1294-5 (1980) (inactivated vaccine) and Sergeev, et al., *Vopr. Virusol.,* 42(5):226-9 (1997) (immunomodifiers ridostin, reaferon, and polyribonate)) are used.

[0242] In another embodiment, an anti-Marburg virus vaccine is used (e.g., the vaccines described in Hevey, et al., *Virology,* 239(1):206-16 (1997) (Baculovirus recombinants were made to express the MBGV glycoprotein (GP) either as a full-length, cell-associated molecule or a slightly truncated (5.4%) product secreted into medium; and killed (irradiated) MBGV antigen)) can be used.

[0243] (c) Anti-Flaviviridae Vaccine

[0244] An anti-Flaviviridae vaccine, such as an anti-Dengue virus vaccine, can be used (e.g., U.S. Pat. No. 5,494,671, Becker, *Virus Genes,* 9(1):33-45 (1994) (Dengue fever virus and Japanse encephalitis virus synthetic peptides with motifs to fit HLA class I haplotypes), Blok, et al., *Virology.,* 187(2):573-90 (1992) (Dengue-2 virus vaccine), Dharakul, et al., *J. Infect. Dis.,* 170(1):27-33 (1994) (live attenuated Dengue virus type 2 vaccine), Green, et al., *J. Virol.,* 67(10):5962-7 (1993) (live attenuated Dengue virus type 1 vaccine), Hoke, et al., *Am. J. Trop. Med. Hyg.,* 43(2):219-26 (1990) (attenuated Dengue 4 (341750 Carib) virus vaccine), Khin, et al., *Am. J. Trop. Med. Hyg.,* 51(6):864-9 (1994), (Dengue-2 PDK53 candidate vaccine), Kinney, et al., *Virology.,* 230(2):300-8 (1997) (attenuated vaccine derivative, strain PDK-53), Leblois, et al., *Nucleic Acids Res.,* 21(7):1668 (1993) (Dengue virus type 2 (strain PR-159) NS1 gene and its vaccine derivative), Marchette, et al., *Am. J. Trop. Med. Hyg.,* 43(2):212-8 (1990) (attenuated Dengue 4 (341750 Carib) virus vaccine), Price, et al., *Am. J. Epidemiol.,* 94(6):598-607 (1971) (injection with Dengue virus), Putnak, et al., *Am. J. Trop. Med. Hyg.,* 55(5):504-10 (1996) (purified, inactivated, Dengue-2 virus vaccine prototype made in fetal rhesus lung cells), Putnak, et al., *J. Infect. Dis.,* 174(6):1176-84(1996) (purified, inactivated,

Dengue-2 virus vaccine prototype in Vero cells), Schlesinger, et al., *J Gen Virol.*, 68(3):853-7 (1987) (Dengue 2 virus non-structural glycoprotein NS1)).

[0245] (d) Anti-Arenaviridae Vaccine

[0246] Anti-Arenaviridae vaccine such as, an anti-Junin virus vaccine (e.g., vaccines described in Boxaca, et al., *Medicina (BAires)*, 41(4):25-34 (1981) (Variant XJO of Junin virus), Contigiani, et al., *Acta Virol.*, 37(1):41-6 (1993) (Candid 1 attenuated strain of Junin virus), Coto, et al., *J Infect Dis.*, 141(3):389-93 (1980) (Protection of guinea pigs inoculated with Tacaribe virus against lethal doses of Junin virus), de Guerrero, et al., *Acta Virol.*, 29(4):334-7 (1985) (attenuated XJO Junin virus (JV) strain), Ghiringhelli, et al., *Am J Trop Med Hyg.*, 56(2):216-25 (1997) (Junin virus vaccine strain (Candid #1), Remesar, et al., *Rev Argent Microbiol.*, 21(3-4):120-6 (1989) (the attenuated XJC13 Junin virus strain), Samoilovich, et al., *Am J Trop Med Hyg.*, 32(4):825-8 (1983) (attenuated XJC13 strain of Junin virus), Videla, et al., *J Med Virol.*, 29(3):215-20 (1989) (Formalin inactivated Junin virus: The XJ-Clone 3 strain of Junin virus) and Weissenbacher, et al., *Intervirology.*, 6(1):42-9 (1975-76) (Tacaribe virus)) can be used.

[0247] An anti-Lassa vaccine can be used (e.g., vaccines described in Auperin, et al., *Virus Res.*, 9(2-3):233-48 (1988) (a recombinant vaccinia virus expressing the Lassa virus glycoprotein gene), Fisher-Hoch, et al., *Proc Natl Acad Sci USA*, 86(1):317-21 (1989) (a recombinant vaccinia virus expressing the Lassa virus glycoprotein gene), Kiley, et al., *Lancet*, 2(8145):738 (1979) (Immunization with closely related Arenavirus), Morrison, et al., *Virology*, 171 (1):1179-88 (1989) (Vaccinia virus recombinants expressing the nucleoprotein or the envelope glycoproteins of Lassa virus)).

[0248] An anti-Machupo virus vaccine (see, e.g., Eddy, et al., *Bull World Health Organ.*, 52(4-6):723-7 (1975)) can be used.

[0249] (2) Anti-viral antibodies

[0250] Anti-viral antibodies can be prepared according to the methods known in the art (see Current Protocols in Immunology (Ed. Coligan et al.) John Wiley & Sons, Inc., 1997). Any types of antibodies, including polyclonal, monoclonal, humanized, Fab fragment, $(Fab)_2$ fragment and Fc fragment, can be used. In a specific embodiment, a monoclonal anti-viral antibody is used. Preferably, the monoclonal antibody is humanized. Also preferably, an IgG or IgM anti-viral antibody is used.

[0251] (a) Anti-Bunyaviridae Antibody

[0252] An anti-Bunyaviridae antibody, such as an anti-Hantaan virus antibody can be used (see, e.g., Kikuchi, et al., *Arch. Virol.*, 143(1):73-83 (1998) (Neutralizing monoclonal antibody (MAb) to envelope protein G1 (16D2) and G2 (11E10)), Liang, et al., *Virology*, 217(1):262-71 (1996) (MAb to G2(HCO2)).

[0253] (b) Anti-Filoviridae Antibody

[0254] An anti-Filoviridae antibody, such as an anti-ebola virus antibody can be used (see, e.g., the following Genbank accession numbers for suitable antigenic proteins: 1EBOA-1EBOF, AAD14582-AAD14590, AAC57989-AAC57993, AAC54882-AAC54891, AAC24345-AAC24346, AAC09342, CAA47483, AAB81001-AAB81007, S23155, VHIWEB, S32584-S32585, AAB37092-AAB37097, AAA96744-AAA96745, AAA79970, CAA43578-CAA43579 and AAA42976-AAA42977, and for nucleic acids: AF086833, U77384-U77385, U8116-U23417, U23187, U23152, U23069, AF034645, AF054908, X67110, L11365, U28077, U28134, U28006, U31033, U23458, X61274, J04337 and M33062).

[0255] An anti-Marburg antibody can be used. The antibodies can be raised against Marburg virus protein sequences with the following Genbank accession numbers are used: AAC40455-AAC40460, VHIWMV, RRIWMV, S44052-S44053, S33316, S32582-S32583, A45705, B45705, S44049, S44054, CAA78114-CAA78120, CAA82536-CAA82542, CAA45746-CAA45749, CAA48507-CAA48509 and AAA46562-AAA46563 or encoded by nucleic acid molecules containing nucleotide sequences with the following Genbank accession numbers are used: AF005730-AF005735, Z12132, Z29337, X64405-X64406, X68493-X68495, M72714, M92834 and M36065.

[0256] (c) Anti-Flaviviridae Antibody

[0257] An anti-Flaviviridae antibody,. such as an anti-Dengue virus antibody is used (see, e.g., Bhoopat, et al., *Asian Pac. J. Allergy Immunol.*, 14(2):107-13 (1996), Hiramatsu, et al., *Virology.*, 224(2):437-45 (1996) (mAb3H5), Roehrig, et al., *Virology*, 246(2):317-28 (1998) (Murine monoclonal antibodies (MAbs) specific for the envelope (E) glycoprotein of DEN 2 virus: Domains A and B), Tadano, et al., *J. Gen. Virol.*, 70 (6):1409-15 (1989) (MAb against the DEN-4 virus core protein Mr 15.5K), Trirawatanapong, et al., *Gene*, 116(2):139-50 (1992) (mAb3H5)).

[0258] (d) Anti-Arenaviridae Antibody

[0259] An anti-Arenaviridae antibody, such as an anti-Junin virus antibody can be used (see, e.g., the antibodies described in Mackenzie, et al., *Am. J. Trop. Med. Hyg.*, 14(6):1079-84 (1965)).

[0260] An anti-Lassa antibody can be used (see, e.g., the antibodies described in Kunitskaia, et al., *Zh Mikrobiol Epidemiol Immunobiol.*, 3:67-70 (1991) and Schmitz, et al., *Med. Microbiol. Immunol. (Berl).*, 175(2-3):181-2 (1986)).

[0261] An anti-Machupo antibody can be used (see, e.g., Mackenzie, et al., *Am. J. Trop. Med. Hyg.*, 14(6):1079-84 (1965)).

[0262] (3) Viral-activated immune cell and serum

[0263] Viral-activated immune cells and sera can be prepared according to the methods known in the art (see Current Protocols in Immunology (Ed. Coligan et al.) John Wiley & Sons, Inc., 1997). Among the cells that can be used for treatment are virally-activated cytotoxic cells (see, Asada, et al., *J. Gen. Virol.*, 68(7):1961-9 (1987) (Adoptive transfer of immune serum or immune T cells for treating Hantaan virus); Nakamura, et al., *J. Infect. Dis.*, 151(4):691-7 (1985) (Immune spleen cells for treating Hantaan virus); Jahrling, et al., *J. Infect. Dis.*, 179(Suppl1):S224-34 (1999) (Hyperimmune equine IgG for treating ebola virus); Mupapa, et al., *J. Infect. Dis.*, 179(Suppl1):S18-23 (1999) (Blood transfusions with blood donated by convalescent patients for treating ebola virus), Avila, et al., *J. Med. Virol.*, 21(1):67-74 (1987) (Immune serum treatment of Junin virus infection),

Blejer, et al., *Intervirology.*, 21(3):174-7 (1984) (Immune serum treatment of Junin virus infection), Lerman, et al., *Rev. Argent. Microbiol.*, 18(1):33-5 (1986) (Homologous hyperimmune serum (HIS) for treating Junin virus), and Jahrling, *J. Med. Virol.*, 12(2):93-102 (1983) (Lassa-immune plasma of guinea pig, primate, and human origin)).

[0264] (4) Small molecule anti-viral agents

[0265] Any small molecule anti-viral agents, when used alone or in combination with other compounds, that can alleviate, reduce, ameliorate, prevent, or place or maintain in a state of remission of clinical symptoms or diagnostic markers associated with viral hemorrhagic diseases or disorders, particularly those viral hemorrhagic diseases or disorders caused by infection of a Bunyaviridae, a Filoviridae, a Flaviviridae, or an Arenaviridae virus, can be used in the present combinations and methods.

[0266] For example, glycyrrhizinic acid and its derivatives for inhibition of Marburg virus reproduction (Pokrovskii, et al., *Dokl Akad Nauk.*, 344(5):709-11 (1995)), Ribavirin (e.g., Ribavirin 2', 3', 5'-triacetate) for Inhibition of Dengue virus (Koff, et al., *Antimicrob. Agents Chemother.*, 24(1):134-6 (1983)), Riba-virin for inhibition of Lassa virus (Jahrling, et al., *J. Infect. Dis.*, 141 (5):580-9 (1980)), and Desferal (e.g., desferrioxamine), Ribavirin for inhibition of Marburg virus (Ignatyev et al., *Voprosy Virusologil*, 41:206-209 (1996) can be used.

[0267] 2. Formulation and routes of administration

[0268] The compounds, blood-derived compositions and agents are preferably formulated as pharmaceutical compositions, preferably for single dosage administration. The concentrations of the compounds in the formulations or the protein concentration of the blood-derived composition are selected to be effective for delivery of an amount, upon administration, that is effective for the intended treatment. Typically, the compositions are formulated for single dosage administration.

[0269] To formulate a composition, the weight fraction of a compound or mixture thereof is dissolved, suspended, dispersed or otherwise mixed in a selected vehicle at an effective concentration such that the treated condition is relieved or ameliorated. Pharmaceutical carriers or vehicles suitable for administration of the compounds provided herein include any such carriers known to those skilled in the art to be suitable for the particular mode of administration.

[0270] Effective concentration of the blood-derived compositions can be empirically determined. Plasma and serum may be administered without further processing or processed according to known methods.

[0271] In addition, the compounds may be formulated as the sole pharmaceutically active ingredient in the composition or may be combined with other active ingredients. Liposomal suspensions, including tissue-targeted liposomes, may also be suitable as pharmaceutically acceptable carriers. These may be prepared according to methods known to those skilled in the art. For example, liposome formulations may be prepared as described in U.S. Pat. No. 4,522,811.

[0272] The active compound is included in the pharmaceutically acceptable carrier in an amount sufficient to exert a therapeutically useful effect in the absence of undesirable side effects on the patient treated. The therapeutically effective concentration may be determined empirically by testing the compounds in known in vitro and in vivo systems, such as the assays provided herein.

[0273] The concentration of active compound in the drug composition will depend on absorption, inactivation and excretion rates of the active compound, the physicochemical characteristics of the compound, the dosage schedule, and amount administered as well as other factors known to those of skill in the art.

[0274] Typically a therapeutically effective dosage The amounts administered may be on the order of 0.001 to 1 mg/ml, preferably about 0.005-0.05 mg/ml, more preferably about 0.01 mg/ml, of blood volume Pharmaceutical dosage unit forms are prepared to provide from about 1 mg to about 1000 mg and preferably from about 10 to about 500 mg, more preferably about 25-75 mg of the essential active ingredient or a combination of essential ingredients per dosage unit form. The precise dosage can be empirically determined.

[0275] The active ingredient may be administered at once, or may be divided into a number of smaller doses to be administered at intervals of time. It is understood that the precise dosage and duration of treatment is a function of the disease being treated and may be determined empirically using known testing protocols or by extrapolation from in vivo or in vitro test data. It is to be noted that concentrations and dosage values may also vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or use of the claimed compositions and combinations containing them.

[0276] Preferred pharmaceutically acceptable derivatives include acids, salts, esters, hydrates, solvates and prodrug forms. The derivative is typically selected such that its pharmacokinetic properties are superior to the corresponding neutral compound.

[0277] Thus, effective concentrations or amounts of one or more of the compounds provided herein or pharmaceutically acceptable derivatives thereof are mixed with a suitable pharmaceutical carrier or vehicle for systemic, topical or local administration to form pharmaceutical compositions. Compounds are included in an amount effective for ameliorating or treating the disorder for which treatment is contemplated. The concentration of active compound in the composition will depend on absorption, inactivation, excretion rates of the active compound, the dosage schedule, amount administered, particular formulation as well as other factors known to those of skill in the art.

[0278] Solutions or suspensions used for parenteral, intradermal, subcutaneous, or topical application can include any of the following components: a sterile diluent, such as water for injection, saline solution, fixed oil, polyethylene glycol, glycerine, propylene glycol or other synthetic solvent; antimicrobial agents, such as benzyl alcohol and methyl parabens; antioxidants, such as ascorbic acid and sodium bisulfite; chelating agents, such as ethylenediaminetetraacetic acid (EDTA); buffers, such as acetates, citrates and

phosphates; and agents for the adjustment of tonicity such as sodium chloride or dextrose. Parenteral preparations can be enclosed in ampules, disposable syringes or single or multiple dose vials made of glass, plastic or other suitable material.

[0279] In instances in which the compounds exhibit insufficient solubility, methods for solubilizing compounds may be used. Such methods are known to those of skill in this art, and include, but are not limited to, using cosolvents, such as dimethylsulfoxide (DMSO), using surfactants, such as Tween®, or dissolution in aqueous sodium bicarbonate. Derivatives of the compounds, such as prodrugs of the compounds may also be used in formulating effective pharmaceutical compositions. For ophthalmic indications, the compositions are formulated in an ophthalmically acceptable carrier. For the ophthalmic uses herein, local administration, either by topical administration or by injection is preferred. Time release formulations are also desirable. Typically, the compositions are formulated for single dosage administration, so that a single dose administers an effective amount.

[0280] Upon mixing or addition of the compound with the vehicle, the resulting mixture may be a solution, suspension, emulsion or other composition. The form of the resulting mixture depends upon a number of factors, including the intended mode of administration and the solubility of the compound in the selected carrier or vehicle. If necessary, pharmaceutically acceptable salts or other derivatives of the compounds may be prepared.

[0281] The compound is included in the pharmaceutically acceptable carrier in an amount sufficient to exert a therapeutically useful effect in the absence of undesirable side effects on the patient treated. It is understood that number and degree of side effects depends upon the condition for which the compounds are administered. For example, certain toxic and undesirable side effects are tolerated when treating life-threatening illnesses that would not be tolerated when treating disorders of lesser consequence. The concentration of compound in the composition will depend on absorption, inactivation and excretion rates thereof, the dosage schedule, and amount administered as well as other factors known to those of skill in the art.

[0282] The compounds can also be mixed with other active materials, that do not impair the desired action, or with materials that supplement the desired action, such as cardiovascular drugs, antibiotics, anticoagulants and other such agents known to those of skill in the art for treating hemorrhagic viral infections, shock, infection, trauma and other disorders for which the treatments provided herein are contemplated.

[0283] Upon mixing or addition of the compound(s), the resulting mixture may be a solution, suspension, emulsion or the like. The form of the resulting mixture depends upon a number of factors, including the intended mode of administration and the solubility of the compound in the selected carrier or vehicle. The effective concentration is sufficient for ameliorating the symptoms of the disease, disorder or condition treated and may be empirically determined.

[0284] The formulations of the compounds and agents for use herein include those suitable for oral, rectal, topical, inhalational, buccal (e.g., sublingual), parenteral (e.g., subcutaneous, intramuscular, intradermal, or intravenous), transdermal administration or any route. The most suitable route in any given case will depend on the nature and severity of the condition being treated and on the nature of the particular active compound which is being used.

[0285] The formulations are provided for administration to humans and animals in unit dosage forms, such as tablets, capsules, pills, powders, granules, sterile parenteral solutions or suspensions, and oral solutions or suspensions, and oil-water emulsions containing suitable quantities of the compounds or pharmaceutically acceptable derivatives thereof. The pharmaceutically therapeutically active compounds and derivatives thereof are typically formulated and administered in unit-dosage forms or multiple-dosage forms. Unit-dose forms as used herein refers to physically discrete units suitable for human and animal subjects and packaged individually as is known in the art. Each unit-dose contains a predetermined quantity of the therapeutically active compound sufficient to produce the desired therapeutic effect, in association with the required pharmaceutical carrier, vehicle or diluent. Examples of unit-dose forms include ampules and syringes and individually packaged tablets or capsules. Unit-dose forms may be administered in fractions or multiples thereof. A multiple-dose form is a plurality of identical unit-dosage forms packaged in a single container to be administered in segregated unit-dose form. Examples of multiple-dose forms include vials, bottles of tablets or capsules or bottles of pints or gallons. Hence, multiple dose form is a multiple of unit-doses which are not segregated in packaging.

[0286] The composition can contain along with the active ingredient: a diluent such as lactose, sucrose, dicalcium phosphate, or carboxymethylcellulose; a lubricant, such as magnesium stearate, calcium stearate and talc; and a binder such as starch, natural gums, such as gum acaciagelatin, glucose, molasses, polvinylpyrrolidine, celluloses and derivatives thereof, povidone, crospovidones and other such binders known to those of skill in the art. Liquid pharmaceutically administrable compositions can, for example, be prepared by dissolving, dispersing, or otherwise mixing an active compound as defined above and optional pharmaceutical adjuvants in a carrier, such as, for example, water, saline, aqueous dextrose, glycerol, glycols, ethanol, and the like, to thereby form a solution or suspension. If desired, the pharmaceutical composition to be administered may also contain minor amounts of nontoxic auxiliary substances such as wetting agents, emulsifying agents, or solubilizing agents, pH buffering agents and the like, for example, acetate, sodium citrate, cyclodextrine derivatives, sorbitan monolaurate, triethanolamine sodium acetate, triethanolamine oleate, and other such agents. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., 15th Edition, 1975. The composition or formulation to be administered will, in any event, contain a quantity of the active compound in an amount sufficient to alleviate the symptoms of the treated subject.

[0287] Dosage forms or compositions containing active ingredient in the range of 0.005% to 100% with the balance made up from non-toxic carrier may be prepared. For oral administration, a pharmaceutically acceptable non-toxic composition is formed by the incorporation of any of the

normally employed excipients, such as, for example pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, talcum, cellulose derivatives, sodium crosscarmellose, glucose, sucrose, magnesium carbonate or sodium saccharin. Such compositions include solutions, suspensions, tablets, capsules, powders and sustained release formulations, such as, but not limited to, implants and microencapsulated delivery systems, and biodegradable, biocompatible polymers, such as collagen, ethylene vinyl acetate, polyanhydrides, polyglycolic acid, polyorthoesters, polylactic acid and others. Methods for preparation of these formulations are known to those skilled in the art.

[0288] For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinized maize starch, polyvinyl pyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets may be coated by methods well-known in the art. The pharmaceutical preparation may also be in liquid form, for example, solutions, syrups or suspensions, or may be presented as a drug product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid).

[0289] Formulations suitable for rectal administration are preferably presented as unit dose suppositories. These may be prepared by admixing the active compound with one or more conventional solid carriers, for example, cocoa butter, and then shaping the resulting mixture.

[0290] Formulations suitable for topical application to the skin or to the eye preferably take the form of an ointment, cream, lotion, paste, gel, spray, aerosol, or oil. Carriers which may be used include vaseline, lanoline, polyethylene glycols, alcohols, and combinations of two or more thereof. The topical formulations may further advantageously contain 0.05 to 15 percent by weight of thickeners selected from among hydroxypropyl methyl cellulose, methyl cellulose, polyvinylpyrrolidone, polyvinyl alcohol, poly (alkylene glycols), poly/hydroxyalkyl, (meth)acrylates or poly-(meth)acrylamides. The topical formulations is most often applied by instillation or as an ointment into the conjunctival sac. It, however, can also be used for irrigation or lubrication of the eye, facial sinuses, and external auditory meatus. It may also be injected into the anterior eye chamber and other places. The topical formulations in the liquid state may be also present in a hydrophilic three-dimensional polymer matrix in the form of a strip, contact lens, and the like from which the active components are released.

[0291] For administration by inhalation, the compounds for use herein can be delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin, for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

[0292] Formulations suitable for buccal (sublingual) administration include lozenges containing the active compound in a flavored base, usually sucrose and acacia or tragacanth; and pastilles containing the compound in an inert base such as gelatin and glycerin or sucrose and acacia.

[0293] The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water or other solvents, before use.

[0294] Formulations suitable for transdermal administration may be presented as discrete patches adapted to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Such patches suitably contain the active compound as an optionally buffered aqueous solution of, for example, 0.1 to 0.2 M concentration with respect to the said active compound. Formulations suitable for transdermal administration may also be delivered by iontophoresis (see, e.g., Pharmaceutical Research 3 (6), 318 (1986)) and typically take the form of an optionally buffered aqueous solution of the active compound.

[0295] In addition to the common dosage forms set out above, the pharmaceutical compositions may also be administered by controlled release means and/or delivery devices such as those described in U.S. Pat. Nos. 3,536,809; 3,598,123; 3,630,200; 3,845,770; 3,847,770; 3,916,899; 4,008,719; 4,687,610; 4,769,027; 5,059,595; 5,073,543; 5,120,548; 5,354,566; 5,591,767; 5,639,476; 5,674,533 and 5,733,566.

[0296] Also provided are combinations for carrying out the therapeutic regimens. Such combinations, which may be packaged in the form of kits, contain one or more containers with therapeutically effective amounts of one or more tetracycline compounds and an anti-viral-hemorrhagic agent, in pharmaceutically acceptable form. The tetracycline compounds and the anti-viral-hemorrhagic agent, either separately or in a mixture, may be in the form of a pharmaceutically acceptable solution, e.g., in combination with sterile saline, dextrose solution, or buffered solution, or other pharmaceutically acceptable sterile fluid. Alternatively, the tetracycline compound and the anti-viral-hemorrhagic agent, either separately or in a mixture, may be lyophilized or desiccated; in this instance, the kit optionally further comprises in a container a pharmaceutically acceptable solution (e.g., saline, dextrose solution, etc.), preferably sterile, to reconstitute the tetracycline compound and the anti-viral-hemorrhagic agent to form a solution for injection purposes.

[0297] In another embodiment, a kit further comprises a needle or syringe, preferably packaged in sterile form, for

injecting the complex, and/or a packaged alcohol pad. Instructions are optionally included for administration of the tetracycline compound and the anti-viral-hemorrhagic agent by a clinician or by the patient.

[0298] The magnitude of a therapeutic dose of the tetracycline compound(s), alone or in combination with the anti-viral-hemorrhagic agent will vary with the severity of the condition to be treated and the route of administration. The dose, and perhaps dose frequency, will also vary according to the age, body weight, condition and response of the individual patient. Dosage and administration may be empirically determined.

[0299] Desirable blood levels may be maintained by a continuous infusion of the tetracycline compound(s) and/or the anti-viral-hemorrhagic agent as ascertained by plasma levels. It should be noted that the attending physician would know how to and when to terminate, interrupt or adjust therapy to lower dosage due to toxicity, or bone marrow, liver or kidney dysfunctions. Conversely, the attending physician would also know how to and when to adjust treatment to higher levels if the clinical response is not adequate (precluding toxic side effects).

[0300] The efficacy and/or toxicity of the tetracycline compound(s), alone or in combination with the anti-viral-hemorrhagic agent can also be assessed by the methods known in the art, i.e., in animal models and/or clinical studies. For example, the efficacy and/or toxicity can be assessed in the animal models described in the following literatures: Huggins et al., *J. Infect. Dis.*, 179(Suppl):S240-247 (1999) (ebola virus leathal mouse model); Lupton et al., *Lancet*, 2(8207):1294-5 (1980) (ebola virus guineapig model); Johnson et al., *J. Virol.*, 73(1):783-786 (1999) (Dengue virus mouse model); Campetella et al., *J. Med. Virol.*, 26(4):443-51 (1988) (Junin virus murine model); de Guerreol et al., *J. Med. Virol.*, 15(2):197-202 (1985) (Junin virus guineapig model); Boxaca et al., *Acta Virol.*, 28(3):198-203 (1984) (Junin virus guineapig model); Blejer et al., *Medicina (B Aires)*, 43(6Pt2):898 (1983) (Junin virus rat model); and Frigerio et al., *Medicina (B Aires)*, 38(5):603-4 (1978) (experimental model in Argentinean hemorrhagic fever).

[0301] Any suitable route of administration may be employed for providing the patient with an effective dosage of the tetracycline compound(s), alone or in combination with the anti-viral-hemorrhagic agent. For example, oral, transdermal, iontophoretic, parenteral (subcutaneous, intramuscular, intrathecal and the like) may be employed. Dosage forms include tablets, troches, cachet, dispersions, suspensions, solutions, capsules, patches, and the like. (See, *Remington's Pharmaceutical Sciences*).

[0302] The active compounds or pharmaceutically acceptable derivatives may be prepared with carriers that protect the compound against rapid elimination from the body, such as time release formulations or coatings.

[0303] Finally, the compounds may be packaged as articles of manufacture containing packaging material, a compound or suitable derivative thereof provided herein, which is effective for treatment of a viral hemorrhagic disease, within the packaging material, and a label that indicates that the compound or a suitable derivative thereof is for treating hemorrhagic diseases or shock or other disorder contemplated herein. The label can optionally include the disorders for which the therapy is warranted.

[0304] E. Blood-derived Compositions and Methods of Treatment

[0305] 1. Blood-derived compositions and processes for producing compositions for treating diseases and disorders characterized by or associated with acute inflammatory responses

[0306] Also provided herein, are methods for preparing blood-derived compositions for treatment of the diseases and disorders characterized by or associated with acute inflammatory responses. The diseases and disorders contemplated herein include, but are not limited to, the viral hemorrhagic fevers, bacterial sepsis, viral hemorrhagic diseases as well as any disorder involving a cytotoxic immune response, including, but not limited to sepsis, cachexia, rheumatoid arthritis, chronic myelogenous leukemia and transplanted bone marrow-induced graft-versus-host disease, septic shock, immune complex-induced colitis, cerebrospinal fluid inflammation, autoimmune disorders, multiple sclerosis and other such disorders that involve release of inflammatory response mediators, including tumor necrosis factor (TNF) interleukins, particularly IL-1, and other interleukins including IL-6 and IL-8, chemokines platelet-activating factor (PAF), prostaglandins and leukotrienes (see, e.g., (1991) *Ann. Intern. Med.* 115: 464-466 for a more comprehensive listing).

[0307] Processes for producing these compositions are provided. The compositions are produced by contacting blood or fraction thereof either in vitro or in vivo with one or more tetracycline or tetracycline-like compounds in an sufficient amount and for a sufficient time to produce a response that is assessed by measuring the level of IL-1 and/or TNF receptors, using any standard assay, and looking for about a 3-fold or greater increase. The resulting blood or composition can be processed further or injected, preferably into a species and blood-type matched mammalian recipient.

[0308] Further processing can be used to isolate fractions thereof that exhibit the anti-inflammatory properties of the unfractionated properties. Fractions include, but are not limited to, the γ-globuline fraction, the AHF (anti-hemophilia factor, the albumin fraction, serum and plasma. Each fraction can be tested in model systems, such as those exemplified herein (see EXAMPLES) to identify active fractions. In addition or alternatively, fractions of interest are those that contain TNF and/or IL-1 receptors. The TNF and IL-1 receptors serve as indicators of the fractions of interest which contain other components that may contribute to the observed effectiveness of the blood-derived fractions in treating the acute inflammatory disorders.

[0309] In one embodiment, the process includes the steps of administering one or more tetracycline or tetracycline-like compound(s) to a mammal; b) collecting blood from the mammal; and c) recovering serum or plasma from the collected blood. Before step a) the baseline level of an indicator of stimulation is obtained. Preferably the level of IL-1 or TNF receptors is assessed, although the level of other cytokines and receptors, such as IL-16 (LCF—chemotactic for CD4, T-lymphocytes), or IL-2 receptors, is assessed using standard methods (i.e., R&D Systems, makes a variety of reagents to test for interleukins and receptors therefor). In

some instances and for certain diseases, cells that produce particular factors may be identified, and those cells stimulated in vitro or in vivo to produce compositions for treatment of those diseases.

[0310] The resulting recovered serum and plasma can be used to administer to mammals exhibiting an acute inflammatory response, such as that associated with infection with a hemorrhagic virus or otherwise exhibiting symptoms of a septic reaction, such as shock, and the other disorders enumerated herein or known to involve a deleterious inflammatory response. The plasma or serum can be further fractionated and tested in model systems to identify active fractions. Any tetracycline or tetracycline-like compound provided herein or known to those of skill in the art is contemplated for use.

[0311] For in vitro preparation, blood or a fraction thereof is contacted with a tetracycline or tetracycline-like compound(s) or other agent, such as a virus, for time sufficient to observe at least a three-fold increase from baseline in the level of TNF or IL-1 receptors. The medium from the blood or fraction is isolated and further processed, such as by further fractionation, or concentration, and then it is administered to a mammal with an acute inflammatory disease, condition or disorder.

[0312] In one embodiment, white cells are harvested from the buffy coat of blood. The cells are treated, for example with Sendei virus to stimulate production of α-interferon, and the supernatant or medium from the cells is isolated. Any process whereby TNF, or IL-1 receptors can be generated, in vitro or in vivo can be used, and the resulting blood product or a derivative thereof administered.

[0313] a. Preparation of Serum and Plasma

[0314] Serum or plasma can be recovered from the collected blood by any methods known in the art. In one specific embodiment, the serum or plasma is recovered from the collected blood by centrifugation. Preferably, the centrifugation is conducted in the presence of a sealant having a specific gravity greater than that of the serum or plasma and less than that of the blood corpuscles which will form the lower, whereby upon centrifugation, the sealant forms a separator between the upper serum or plasma layer and the lower blood corpuscle layer. The sealants that can be used in the processes include, but are limited to, styrene resin powders (Japanese Patent Publication No. 38841/1973), pellets or plates of a hydrogel of a crosslinked polymer of 2-hydroxyethyl methacrylate or acrylamide (U.S. Pat. No. 3,647,070), beads of polystyrene bearing an antithrombus agent or a wetting agent on the surfaces (U.S. Pat. No. 3,464,890) and a silicone fluid (U.S. Pat. Nos. 3,852,194 and 3,780,935). In a preferred embodiment, the sealant is a polymer of unsubstituted alkyl acrylates and/or unsubstituted alkyl methacrylates, the alkyl moiety having not more than 18 carbon atoms, the polymer material having a specific gravity of about 1.03 to 1.08 and a viscosity of about 5,000 to 1,000,000 cps at a shearing speed of about 1 second^{-1} when measured at about 25° C. (U.S. Pat. No. 4,140,631).

[0315] In another specific embodiment, the serum or plasma is recovered from the collected blood by filtration. Preferably, the blood is filtered through a layer of glass fibers with an average diameter of about 0.2 to 5μ and a density of about 0.1 to 0.5 g/cm^3, the total volume of the plasma or serum to be separated being at most about 50% of the absorption volume of the glass fiber layer; and collecting the run-through from the glass fiber layer which is plasma or serum (U.S. Pat. No. 4,477,575). Also preferably, the blood is filtered through a layer of glass fibers having an average diameter 0.5 to 2.5μ impregnated with a polyacrylic ester derivative and polyethylene glycol (U.S. Pat. No. 5,364, 533). More preferably, the polyacrylic ester derivative is poly(butyl acrylate), poly(methyl acrylate) or poly(ethyl acrylate), and (a) poly(butyl acrylate), (b) poly(methyl acrylate) or poly(ethyl acrylate) and (c) polyethylene glycol are used in admixture at a ratio of (10-12):(1-4):(1-4). In still another specific embodiment, the serum or plasma is recovered from the collected blood by treating the blood with a coagulant containing a lignan skelton having oxygen-containing side chains or rings (U.S. Pat. No. 4,803,153). Preferably, the coagulant comprises a lignan skelton having oxygen-containing side chains or rings, e.g., d-sesamin, I-sesamin, paulownin, d-asarinin, I-asarinin, 2α-paulownin, 6α-paulownin, pinoresinol, d-eudesmin, I-pinoresinol β-D-glucoside, I-pinoresinol, I-pinoresinol monomethyl ether β-D-glucoside, epimagnolin, lirioresinol-B, syringaresinol (dl), lirioresinonB-dimethyl ether, phillyrin, magnolin, lirioresinol-A, 2α, 6α-d-sesamin, d-diaeudesmin, lirioresinol-C dimethyl ether (ddiayangambin) and sesamolin. More preferably, the coagulant is used in an amount ranging from about 0.01 to 50 g per 1 L of the blood.

[0316] b. Further Fractionation of Plasma

[0317] Blood plasma or sera can be further separated into different fractions, including, inter alia, an albumin-containing fraction, a globulin-containing fraction and an AHF-containing fraction. Methods for preparing these fractions are known in the arts. Generally, these methods comprise one or more of the following procedures: (a) fractional precipitation with ammonium sulfate and similar salts; (b) organic solvent precipitation with cold ethanol or acetone and other such alcohols and ketones; (c) selective adsorption on calcium phosphate gels or with barium sulfate; (d) isoelectric precipitation by pH adjustment to the point at which there is no net charge on a given protein; and (e) chromatography by use of adsorbents such as CM- or DEAE-cellulose or by "Sephadex" gel filtration. Other procedures for selectively fractionating and purifying blood proteins involve the use of amino acids such as glycine and beta alanine, water-soluble organic polymers such as polyethylene glycol and polypropylene glycol, and water-insoluble polyelectrolyte polymers containing basic amino groups such as the dimethylaminopropylimide group.

[0318] (1) Preparation of Albumin-Containing Fraction

[0319] The plasma can further be separated into a fraction containing albumin by any methods known in the art. In one specific embodiment, the albumin-containing fraction is prepared by selective precipitation with block copolymers of ethylene oxide and polyoxypropylene polymer from the plasma (U.S. Pat. No. 4,025,500).

[0320] In another specific embodiment, the albumin-containing fraction is prepared by: (a) diluting the plasma in liquid form with a NaCl solution containing disodium ethylene dinitrilo tetraacetate and an albumin stabilizer; (b) adjusting the pH of the plasma solution resulting from step (a) to about 6.2; (c) heating the plasma solution from step (b)

at about 60° C. for about 1½ hours; (d) cooling the plasma solution to about 10° C.; (e) precipitating impurities from the solution with polyethylene glycol at a concentration of about 18-20% with the albumin remaining in the supernatant; (f) isoelectrically precipitating albumin from the supernatant at a pH of about 4.6; and (g) recovering the albumin-containing fraction (U.S. Pat. No. 4,164,496). Preferably, the albumin stabilizer is sodium caprylate.

[0321] In still another specific embodiment, the albumin-containing fraction is prepared by: (a) adjusting the pH of the plasma in liquid form to about 6.7; (b) heating the plasma at about 60° C. for about 1½ hours; (c) adjusting the pH of the plasma to about 5.7; (d) precipitating impurities from the plasma by the addition of ethanol in an amount sufficient to give a final concentration of about 40 to 44% in the plasma along with cooling of the plasma to about -5° C., with the albumin remaining in the supernatant; and (e) precipitating albumin-containing fraction from the supernatant at a pH of about 4.8. (U.S. Pat. No. 4,222,934).

[0322] A blood group substance can be removed from the albumin-containing fraction. It can be removed for example, by treating the albumin-containing fraction with polyethylene glycol at pH of about 6.6 to 8.0, the effective polyethylene glycol concentration in the aqueous albumin solution being about 13 to 20% (w/v), in the presence of an inorganic salt at a concentration of at most 50 g/liter measured as sodium chloride and at a temperature in the range of about 2° C. to 30° C., the resulting polyethylene glycol/albumin solution having a protein concentration of about 5 to 40 g/liter, thereby precipitating and removing contaminant proteins containing the blood-group substance (U.S. Pat. No. 4,197,238).

[0323] Alternatively, the a blood group substance can be removed from the albumin-containing fraction by treating the albumin-containing fraction with polyethylene glycol at pH of about 8.0 to 9.6, the effective polyethylene glycol concentration in the aqueous albumin solution being about 15 to 30% (w/v), in the presence of an inorganic salt at a concentration of at most 50 g/liter measured as sodium chloride and at a temperature in the range of about 2° C. to 30° C., the resulting polyethylene glycol/albumin solution having a protein concentration of about 5 to 40 g/liter, thereby precipitating and removing contaminant proteins containing the blood-group substance (U.S. Pat. No. 4,197, 238).

[0324] In another alternative method, the steps for removing a blood group substance from the albumin-containing fraction include treating the albumin-containing fraction with polyethylene glycol having an average molecular weight in the range of about 2,000 to 10,000 at pH of about 6.6 to 9.6, the effective polyethylene glycol concentration in the aqueous albumin solution being about 13 to 20% (w/v), in the presence of an inorganic salt at a concentration of at most 50 g/liter measured as sodium chloride and at a temperature in the range of about 2° C. to 30° C., the resulting polyethylene glycol/albumin solution having a protein concentration of about 5 to 40 g/liter, thereby precipitating and removing contaminant proteins containing the blood-group substance (U.S. Pat. No. 4,197,238).

[0325] Polymer content and α1-AGP content can be reduced in the albumin-containing fractiom such as by subjecting the albumin-containing fraction to ion exchange separation using an anion exchanger, the anion exchange separation is carried out at a pH ranging from about 5.1 to 5.5 (U.S. Pat. No. 5,277,818).

[0326] (2) Preparation of Globulin-Containing Fraction

[0327] The globulin-containing fraction can be prepared according to any methods known in the art. For example, conventional methods such as Cohn alcohol fractionating process (Kistler et al. (1962) *Vox Sang*, 7:414); and Cohn et al. (1946) *J. Am. Chem. Soc.* 68:459-475) and the Rivanol ammonium sulfate fractionation (Horejsi et al. (1956) *Acta Med. Scand.* 155: 65) can be used.

[0328] Alternatively, other known methods can be used (see, e.g., U.S. Pat. Nos. 4,347,138 and 5,310,877). U.S. Pat. No. 4,347,138 describes a method of separating serum albumin and a serum γ-globulin from each other in a solution using a semipermeable membrane by forcing the blood serum protein mixture solution through an ultrafiltration membrane having a cut off molecular weight of about 100,000 and composed of an aromatic polyether sulfone, while adjusting the total protein concentration and salt concentration in the mixture solution to not more than 4 g/dl and not more than 0.6 mole/l, respectively, and also adjusting the pH of the solution to a value of from about 3.8 to about 4.7. Preferably, the pH of the blood serum protein mixture solution is adjusted to a value of from 3.9 to 4.3. Also preferably, the salt contained in the blood serum protein mixture solution is sodium chloride or other physiologically acceptable salt.

[0329] U.S. Pat. No. 5,310,877 describes a method for the separation of gamma globulin from albumin contained in an aqueous solution of both by ultrafiltration using a microfilter having a water permeability of 0.2-25 gallons per square foot per day per pound per square inch including a porous solid filter substrate one surface of which is impregnated with particulate solids affixed within the pores of the substrate having an average particle size of about 0.1-0.5 micrometer at the feed interface, the aqueous solution being characterized in that the total concentration of protein in the aqueous solution is about 0.1-2% by weight, the pH of the aqueous solution is 8-10 and the solution contains no more than about 0.01 mole per liter of inorganic electrolyte, the albumin being enriched in the retentate and the gamma globulin being enriched in the permeate. Preferably, the particulate solids being used are titanium oxide particles. Also preferably, the substrate being used is sintered stainless steel.

[0330] Since intravenous administration is more direct and efficient, it is sometimes desirable or necessary to administer the globulin-containing fraction intravenously. A globulin-containing fraction prepared by the conventional fractionation contains anti-complement activity, i.e., the property of fixing complement non-specifically (U.S. Pat. No. 4,082, 734). This anti-complement activity is related to the formation of aggregates. Such globulin-containing fraction containing the anti-complement activity is not suitable for intravenous administration because the fraction can cause shock in some patients (U.S. Pat. No. 4,124,576). Therefore, the anti-complement activity must be eliminated or reduced before the globulin-containing fraction can be administered intravenously.

[0331] The anti-complement activity can be eliminated or reduced according to any methods known in the art. For

example, pepsin decomposition (Schultze and Schwick, *Dtsch. Med. Wochenschrift*, 87:1643 (1962)); decomposition (Barandun, et al., *Vox Sang.*, 28:157 (1975)); HCl treatment (Barandun, et al., *Vox Sang.*, 7:187 (1962)) and β-propiolactone treatment (Stephan, *Z. Klin. Chem. Klin. Biochemie*, 7:282 (1969)) can be used. In other specific embodiments, the processes described in U.S. Pat. Nos. 4,082,734, 4,075,193, 4,124,576, 4,154,819, 4,374,763, 4,436,724, 4,835,257

[0332] U.S. Pat. No. 4,082,734 describes a method of preparing an intravenously applicable globulin of substantially unchanged half-life but free from anti-complement activity, by heating plasma or serum for about 2 to 4 hours at about 50° C. to 56° C., and then fractionating, the heating having been long enough within the recited parameters so that the product upon fractionation is substantially free from anti-complement activity. Preferably, the fractionation is effected with alcohol or ammonium sulfate. Also preferably, the heating is effected for about 2 hours at about 56° C.

[0333] U.S. Pat. No. 4,075,193 describes a process for producing globulin for intravenous administration which comprises: 1) adsorbing plasminogen derived from blood of a selected mammalian species on an adsorbent substrate of L-lysine agarose; 2) washing the adsorbate to elute impurities; 3) eluting the purified plasminogen from the substrate; 4) converting the eluted plasminogen to plasmin; 5) incubating a mixture of the plasmin and a quantity of homospecific immune globulin having anticomplementary activity under conditions such that the anticomplementary activity is substantially reduced; and 6) inactivating plasmin present in the mixture by adsorption on an inactivation adsorbent for plasmin, and recovering the immune globulin.

[0334] U.S. Pat. No. 4,124,576 describes a process for preparing a gamma globulin substantially devoid of anticomplementary activity and suitable for intravenous administration, from a material selected from the Cohn Fraction II+III plasma protein paste having a protein content of about 25-30%, Cohn Fraction II paste and placental extracts containing these fractions which comprises the steps: 1) suspending the paste in water to form a solution of low ionic strength having a conductance of about 300×10^{-6} cm^{-1} ohm^{-1} at a pH of about 4.9 to 6.0 to produce a precipitate and a filtrate; 2) precipitating impurities from the filtrate by adding polyethylene glycol to 4% (w/v); 3) further precipitating impurities by the addition of ethanol in a concentration of from 4 to 12% (w/v); and 4) precipitating the gamma globulin by adding polyethylene glycol to 10 to 12% (w/v) or by adding ethanol to 20 to 30% (v/v), preferably 25% (v/v) at a pH of from 7 to 8.2, preferably 8.0, the process being carried out at a temperature of about 0-6° C.

[0335] U.S. Pat. No. 4,154,819 describes a process for preparing a γ-globulin solution suitable for the intravenous application by treating the solution of γ-globulin with acetimido ethyl ester hydrochloride, diketene, formimido ethyl ester hydrochloride or propanesultone at a pH of about 9, thereafter adjusting the pH to about 7 to 7.5, and separating the solution from the solids by dialysis or fractionation followed by sterile filtration. Preferably, the diketene is employed in about 0.02 g per g of protein in the γ-globulin solution. U.S. Pat. No. 4,374,763 describes a process for producing γ-globulin suitable for use in intravenous administration and of an anticomplementary activity of lower than 20% by bringing Cohn's Fraction II for the gamma-globulin into suspension in an aqueous solution of a monosaccharide, disaccharide or sugar alcohol, adjusting the pH of the suspension to about 7.0 to 9.0, adding dextran of an average molecular weight of 10,000 to 70,000 into the suspension to produce an aqueous about 2 to 10% (w/v) solution of dextran, and after removing the thus formed precipitate, adding ammonium sulfate to the mother liquor to precipitate the gamma-globulin.

[0336] U.S. Pat. No. 4,436,724 describes a method for producing γ-globulin which can be administered intravenously without adverse reactions. The method includes treating γ-globulin with pepsin or uropepsin in a neutral pH range of about 6.0 to 7.5. The aggregates in γ-globulin are selectively decomposed, while any decomposition of monomer γ-globulin molecule is substantially prevented. The globulin-containing fraction thus produced with reduced anti-complementary activity is stabilized by adding uropepsin which serves simultaneously as a proteolytic enzyme and a stabilizer.

[0337] U.S. Pat. No. 4,835,257 describes a process for the preparation of gamma globulin suitable for intravenous administration. The process includes the steps of: dissolving gamma globulin precipitated from blood or blood products in a solution, separating non-dissolved precipitate from the solution, adding polyethylene glycol to the separated solution, separating precipitate from the polyethylene glycol solution, increasing the polyethylene glycol concentration in the solution, separating precipitated purified gamma globulin from the higher concentrated polyethylene glycol solution, dissolving the purified gamma globulin in a solution suitable for intravenous administration. The process also includes a step of dissolving the gamma globulin precipitated from blood in a solution having a neutral pH, adding polyethylene glycol in the first step to a concentration of about 4.0-5.5% by weight, and increasing the polyethylene glycol concentration in the second step to at least 9% but not more than 16% by weight, and by adding a buffer to the solution just prior to adding the polyethylene glycol in one of the two polyethylene glycol addition steps.

[0338] In another specific embodiment, the globulin-containing fraction can be lyophilized for extended shelf-life and ease of transportation. The globulin-containing fraction can be lyophilized by any methods known in the art, preferably in the presence of salts or sugars. For example, the processes described in the U.S. Pat. Nos. 4,168,303 and 4,692,331 can be used.

[0339] U.S. Pat. No. 4,168,303 describes a process for producing a lyophilized gamma globulin preparation for intravenous administration, which comprises freeze-drying an aqueous solution of gamma globulin which has undergone no modification and has an anticomplementary activity of 20 (CH50) or less in the presence of about 0.06 to 0.26 part by weight of sodium chloride for 1 part by weight of the gamma globulin. Preferably, the freeze drying is carried out in the presence of about 0.1 to 0.3 part by weight of serum albumin for 1 part by weight of the gamma globulin. Also preferably, the freeze drying is carried out in the presence of about 0 to 0.5 part by weight of a diluent for about 1 part by weight of the gamma globulin. Further preferably, the diluent is mannitol.

[0340] U.S. Pat. No. 4,692,331 describes a process for preparing a storage-stable, intravenously administrable

γ-globulin dry preparation, which γ-globulin has been obtained by fractionating plasma with polyethylene glycol and has been substantially freed of remaining polyethylene glycol. The process includes the steps of: (1) adding glucose to an aqueous solution of γ-globulin, which is substantially free of remaining polyethylene glycol and is suitable for intravenous administration, the amount of glucose added being from about 0.2 to 2.0 parts by weight, based on one part of γ-globulin sufficient to stabilize the γ-globulin; and thereafter (2) lyophilizing the aqueous solution to produce a dry powder. Preferably, the aqueous solution contains γ-globulin in an amount of about 5 to 20% (W/V) in terms of protein.

[0341] (3) Preparation of AHF-Containing Fraction

[0342] Factor VIII and von Willebrand's factor are associated plasma proteins that together are called Antihemophilic Factor (AHF). Both are important in the blood clotting mechanism. Methods of making concentrates of AHF are known in the art. These range from simply freezing and then thawing plasma (cryoprecipitation) to yield a more concentrated insoluble mixture of Factor VIII, fibrinogen, cold-insoluble globulin to more involved procedures (e.g., Pool et al. *New England Journal of Medicine,* 273:1443-1447 (1965)). These concentrates may be made more highly purified by further treatment employing techniques such as aluminum hydroxide absorption, glycine extraction, polyethylene glycol concentration, and filtration. The AHF-containing fraction can be prepared according to the above described processes. Alternatively, the processes described in the U.S. Pat. Nos. 3,631,018, 3,652,530, 3,682,881, 3,973,002, 4,069,216 4,089,944, 4,104,266, 4,170,639, 4,203,891, 4,210,580, 4,251,437, 4,289,691, 4,348,315, 4,383,989, 4,386,068, 4,404,131, 4,435,318, 4,522,751, 4,543,210, 4,743,680, 4,814,435, 4,952,675, 4,977,246, 5,484,890, H1,509 and Re. 29,698 can be used.

[0343] U.S. Pat. No. 3,631,018 describes a method for preparing a concentrate of AHF including fractionating a cryoprecipitate concentrate of AHF with polyethylene glycol and glycine in a three-step precipitation: (1) first with about 3-4% by weight of polyethylene glycol followed by recovery of the supernate; (2) then with polyethylene glycol added to about 10% by weight followed by recovery of the resulting precipitate; and (3) finally with about 1.3-1.8 M glycine added to a solution of the precipitate from step (2) followed by recovery of the resulting precipitate. The polyethylene glycol suitable for use in the method has a molecular weight in the range of 200-20,000, preferably 400-6,000, more preferably about 4,000.

[0344] U.S. Pat. No. 3,652,530 describes a method of preparing highly purified AHF by treating an extract of a precipitate obtained by cryoethanol precipitation with polyethylene glycol in three successive precipitations, first with aluminum hydroxide gel at pH about 5.6-7.0, then with polyethylene glycol to a concentration of about 3.0-6.5%, and finally with added polyethylene glycol to a concentration of 10-12% to obtain a precipitate containing the highly purified AHF.

[0345] U.S. Pat. No. 3,682,881 describes a method for the preparation of a prothrombin complex and an AHF concentrate from citrated blood plasma treated with 1.5-1.8 M glycine. The resulting precipitate was treated successively with polyethylene glycol, first to a concentration of 3-4% and then 10% by weight, and finally with 1.8 M glycine.

[0346] U.S. Pat. No. 3,973,002 describes a method for isolating antihemophilic factor of human blood plasma including the steps of adjusting the pH of a solution of buffer-extracted plasma cryoprecipitate to from about 6.0 to about 7.0, and cooling the solution at a temperature of from about 2° C. to about 20° C. for from about 15 to about 60 minutes to cause precipitation of impurities.

[0347] U.S. Pat. No. 4,069,216 describes an improvement in the process described in U.S. Pat. No. 3,631,018 mentioned above, in which the process includes the step of holding a buffered solution of F. VIII and 6% polyol at 0-5° C. until precipitation occurs.

[0348] U.S. Pat. No. 4,089,944 describes a method for producing a clinically useful freeze-dried solid composition containing AHF and fibrinogen from blood plasma or an AHF-containing fraction thereof including the steps of fractionating the plasma to obtain a solid mixture containing AHF and fibrinogen, dissolving the solid mixture in an aqueous medium and freeze-drying the resulting solution to obtain a clinically useful freeze-dried solid composition which is then reconstituted in a reconstitution liquid for use, and including the step of rendering the freeze-dried, solid composition rapidly soluble in an aqueous medium at room temperature by adding water soluble carbohydrate to the mixture, the amount of carbohydrate added being an amount sufficient to produce at least about 2 grams per 100 milliliters concentration of carbohydrate upon reconstitution of the composition in a suitable medium to produce a therapeutically useful solution of AHF. Preferably, the carbohydrate used is dextrose, maltose, lactose or sucrose.

[0349] U.S. Pat. No. 4,104,266 describes a method for the preparation of purified AHF which includes the thawing of frozen plasma at a temperature of between about 0° C. and about 1° C. to obtain a cryoprecipitate containing AHF, and including the steps of: (a) extracting the cryoprecipitate with a low ionic strength buffer solution containing tris (hydroxymethyl) aminomethane at a temperature of about 0° C. to obtain a cold insoluble fraction having cold soluble impurities removed therefrom; (b) extracting the cold insoluble fraction with a low ionic strength buffer solution containing tris (hydroxymethyl) aminomethane at a temperature of about 21° C. to obtain a solution containing AHF and the buffer solution; (c) deprothrombinizing the solution with aluminum hydroxide gel; and (d) recovering an AHF-rich solution.

[0350] U.S. Pat. No. 4,170,639 describes a process for the production of antihemophilic factor concentrate in purified form having enhanced potency and solubility by: (a) subjecting an aqueous extract of antihemophilic blood plasma cryoprecipitate to purification by mixing with an aluminum hydroxide adsorbent at an acid pH and precipitating unwanted protein in the cold, the pH conditions being such that unwanted protein is selectively removed by adsorption without substantial loss of antihemophilic factor potency from the aqueous extract; (b) constituting the purified aqueous extract with buffer and saline and adjusting to an acid pH, and (c) freeze-drying the thus adjusted aqueous extract.

[0351] U.S. Pat. No. 4,203,891 describes a method of increasing the yield of antihemophilic factor VIII (AHF),

from whole blood, blood plasma or blood plasma fractions by collecting the blood or plasma or plasma fraction from a donor directly into an anticoagulant agent selected from heparin, sodium heparin, or mixtures thereof, which agent does not reduce the physiological concentration of calcium, and recovering the AHF. Preferably, the anticoagulant is used in the range of 0.1-10 units/ml based on total volume of whole blood or blood plasma and the AHF is recovered by fractionation using glycine, ethanol, ethanolglycine, polyethylene glycol or glycine-polyethylene glycol precipitation.

[0352] U.S. Pat. No. 4,210,580 describes a process for separating and isolating AHF and fibronectin from plasma by cryoprecipitation (0-15° C.) in the presence of a sulfated mucopolysaccharide, e.g., heparin, to a concentration of about 0.15-0.25 mg/ml of plasma (approximately 22.5 to 37.5 units of heparin/ml of plasma). The resulting fibronectin precipitate is purified chromatographically and the heparin supernatant is mixed with an anion exchange resin such as DEAE cellulose with Heparasorb to remove heparin and to provide a supernatant having 90-95% of the original procoagulant activity.

[0353] U.S. Pat. No. 4,251,437 describes a process for producing an antihemophilic factor preparation (AHF) by thawing deep-frozen human blood plasma, at least partially, by irradiation with electromagnetic waves of a frequency of about 10^8-10^{15} Hz for a period of time and with an energy penetration such that the temperature in the thawed blood plasma does not exceed 10° C. at any point, centrifuging the thawed product to form a cryoprecipitate, redissolving the cryoprecipitate in a buffer, isolating a concentrated solution, and optionally freeze-drying the concentrated solution. Preferably, the irradiation is controlled so that the temperature in the thawed product does not exceed 4° C. at any point. Also preferably, the irradiation is carried out with microwaves of a frequency of about 10^8-3×10^{11} Hz. Further preferably, the irradiation is carried out with microwaves of a frequency of about 2×10^9-3×10^{10} Hz.

[0354] U.S. Pat. No. 4,289,691 describes a method for obtaining AHF from fresh blood plasma by adding heparin, used in the range of about 1-10 units/ml of plasma, to fresh plasma collected by plasmapheresis into a calcium chelating anticoagulant, freezing the plasma, resolubilizing the plasma, isolating a cryoprecipitate from the plasma, resolubilizing the cryoprecipitate, adding a citrate saline heparin buffer to the resolubilized cryoprecipitate, incubating the resolubilized, buffered cryoprecipitate at about 0-10° C. for a time in excess of about 1 hour in the presence of heparin precipitable cold insoluble globulin, separating an AHF rich precipitate and isolating AHF from the precipitate.

[0355] U.S. Pat. No. 4,348,315 describes a process for purifying and/or concentrating the F. VIII complex, starting from cryoprecipitate or Cohn Fraction I-O, by dissolving a composition containing F. VIII together impurities in 1.5 M glycine solution at 15° C. and pH 6.3-7.8 to obtain a solution containing F. VIII and a precipitate containing the impurities. Optionally, the process includes the additional step of adding PEG to the resulting F. VIII-containing glycine solution followed by precipitating and then concentrating purified F. VIII from the solution.

[0356] U.S. Pat. No. 4,383,989 describes a method of obtaining AHF by collecting freshly obtained plasma or plasma fractions directly into heparin, sodium heparin or mixtures thereof, in a proportion of about 6-8 units of heparin/ml of plasma, in the absence of a citrate buffer, and applying a cold incubation technique (0-10° C.) using heparin precipitable cold insoluble globulin.

[0357] U.S. Pat. No. 4,386,068 describes a process for producing an AHF concentrate by treating an aqueous suspension of cryoprecipitate containing AHF proteins with aluminum hydroxide gel, subjecting the resulting solution to ultrafiltration, and then constituting the solution resulting from the ultrafiltration in buffer and saline. Optionally, the solution resulting from the ultrafiltration may be treated with 1.6-2.2 M glycine for further purification.

[0358] U.S. Pat. No. 4,404,131 describes a method of producing an AHF concentrate by subjecting an AHF concentrate obtained by conventional fractionation, e.g., cryoprecipitation, to cryoalcohol precipitation.

[0359] U.S. Pat. No. 4,435,318 describes a process for the separation and recovery of Factor VIII, von Willebrand's factor, and Factor V from plasma and plasma derivative streams having a pH normally between about 6 to 8.5 by removing from the blood stream when present substantially all initial turbidity therein, subsequently passing the blood plasma into and out of an apparatus containing one or more semi-permeable membranes which separate the plasma stream from a salt receiving stream thereby decreasing the salt content of the plasma stream between about 45 to 80% to cause the formation of a protein turbidity enriched in Factor VIII, von Willebrand's factor and Factor V, subsequently removing substantially all of the turbidity and maintaining the temperature of the plasma stream during the separation and recovery process in the range of between about 4-40° C., and at substantially its original starting pH level.

[0360] U.S. Pat. No. 4,522,751 describes a method of producing a preparation containing Factor VIII (AHF) from a Factor-VIII-containing plasma fraction, the preparation containing Factor VIII (AHF) having a specific activity of at least 1.5 units of Factor VIII/mg protein, immunoglobulin G (IgG) of from 15 to 30 mg/1000 units of Factor VIII and fibrinogen of from 20 to 40 mg/100 units of Factor VIII, by: (a) dissolving the Factor-VIII-containing plasma fraction in a buffer solution containing a sulfated polysaccharide at a pH value approximately in the neutral range; (b) lowering the pH to a value ranging from 6.0 to 6.4 and adjusting the temperature to between about 0° C. to about 25° C. to precipitate undesired proteins and obtain a Factor-VIII-containing supernatant; (c) adding at least of glycine, sodium chloride and sodium citrate, to the Factor-VIII-containing supernatant to maintain the major part of the immunoglobulins contained in the supernatant in solution; (d) adding a protein precipitating agent to obtain a Factor-VIII-containing precipitate; and (e) dissolving the Factor-VIII-containing precipitate in a solvent to obtain the final product.

[0361] U.S. Pat. No. 4,543,210 describes a process for producing high purity antihemophilic factor concentrate from an antihemophilic factor-containing dispersion or solution isolated from blood plasma or a blood plasma fraction including performing two consecutive precipitations using a combination of precipitants in each precipitation, first a combination of 1-4% by weight, based on weight of solu-

tion, of polyethylene glycol and 0.1-0.2 ml of 1-3%, based on weight of suspension, aluminum hydroxide suspension per gram of protein in the starting dispersion or solution, followed by a combination of added polyethylene glycol to provide a final concentration of 9-13% by weight, based on weight of the resulting solution, and 10-20% by weight of glycine, based on weight of the polyethylene glycol solution, and 10-20% by weight, based on weight of the polyethylene glycol solution, of sodium chloride.

[0362] U.S. Pat. No. 4,743,680 describes a process for purifying a protein that has antihemophilic factor activity by column chromatography in a column behaving predominantly as an ion-exchange chromatography column, including the steps of: (a) equilibrating the chromatography column; (b) loading a sample containing the protein on the column, causing the protein to adsorb onto the column; (c) washing the column; (d) eluting the adsorbed protein from the column by causing it to desorb from the column; (e) recovering the protein in purified form; and also including the step of: adding to the column a substance containing of an effective amount for selectively increasing the electrostatic forces on the surface of the protein and concomitantly decreasing the hydrophobicity of the protein of a hydration additive selected from among sugars and polyhydric alcohols during at least one of the steps (a), (b), and (c) thereby promoting the adsorption of the protein on the column.

[0363] U.S. Pat. No. 4,814,435 describes a method for preparing a Factor VIII (AHF)-containing fraction having a specific activity of at least 2.5 units of Factor VIII/mg protein as well as a portion of immunoglobulin G (IgG) of 10 mg/1000 units of Factor VIII at most, with the risk of transmission of viral or bacterial infections avoided or largely reduced when applied therapeutically or prophylactically. The method includes the steps of: 1) preparing a first solution of a Factor VIII containing plasma fraction including at least one of a heparinoid and a complex compound of heparin and antithrombin III (Atheplex); 2) precipitating and separating undesired proteins from the first solution in the presence of sulfated polysaccharides at a pH of 6.0 to 6.4 and at a temperature of 0-25° C. so as to obtain a purified Factor VIII containing supernatant; 3) treating the purified Factor VIII containing supernatant with a protein precipitating agent selected from ammonium sulfate, ammonium sulfate-glycine, sodium chloride-glycine, sodium sulfate, sodium sulfa te-sodium citrate, ammonium sulfate-sodium citrate, sodium chloride-ammonium sulfate at a concentration of 8 to 35% and a pH of 5.6 to 6.8 so as to precipitate a Factor VIII containing precipitate; 4) dissolving the Factor VIII containing precipitate in a buffer solution so as to obtain a second solution; 5) one of ultrafiltering and dialyzing the second solution, and lyophilizing so as to obtain a lyophilizate; 6) and heat-treating the lyophilizate at a temperature and for a period of time sufficient to inactivate possibly present viruses.

[0364] U.S. Pat. No. 4,952,675 describes a process for purifying a protein having antihemophilic factor activity by column chromatography in a column behaving predominantly as a hydrophobic affinity chromatography column, including the steps of: (a) equilibrating the chromatography column; (b) loading a sample containing the protein on the column, causing the protein to adsorb onto the column; (c) washing the column; (d) eluting the adsorbed protein from the column by causing it to desorb from the column; (e) recovering the protein in purified form; and also including the step of: adding to the column a substance containing an effective amount for selectively increasing the electrostatic forces on the surface of the protein and concomitantly decreasing the hydrophobicity of the protein of a hydration additive selected from among sugars and polyhydric alcohols during the step (d) thereby promoting the desorption of the protein from the column; and subjecting the eluate containing the protein from the step (d) to a second purification using a second column behaving predominantly as an ion-exchange chromatography column prior to the step (e).

[0365] U.S. Pat. No. 4,977,246 describes a method for obtaining an AHF-rich product from human plasma by: (a) thawing freshly frozen human plasma at a temperature of about 6-10° C. to obtain a plasma solution; (b) adding one volume of about 1.20 M to 1.80 M aqueous solution of a precipitating agent selected from the group consisting of sodium citrate, potassium citrate and citric acid to two volumes of the plasma solution obtained in step (a) at a temperature of about 0-10° C. to form a precipitate; (c) incubating the precipitate-containing solution in an ice bath for about 20 to 40 minutes; and (d) separating the precipitate from the solution.

[0366] U.S. Pat. No. 5,484,890 describes a method of recovering, from a biological sample, an antihemophilic factor protein containing fraction having increased antihemophilic factor protein stabilit. The sample contains (a) an antihemophilic factor protein, (b) at least one destabilizing protease impurity, and (c) at least one proprotease impurity; and the fraction having at least 17 units of antihemophilic factor protein/mg of total protein; the method comprising: contacting the sample with an amount of a protease removing agent effective to remove a destabilizing amount of the protease impurity and an amount of proprotease removing agent effective to remove a destabilizing amount of the proprotease impurity. The proprotease removing agent includes an anion exchange resin in an amount ranging from 70 mg total loading protein/ml anion exchange resin to 750 mg total loading protein/ml anion exchange resin. U.S.

[0367] Patent No. H1,509 describes a process for producing a Factor VIII concentrate from blood plasma, by: (a) obtaining a cryoprecipitate containing Factor VIII from blood plasma; (b) dissolving the cryoprecipitate in an aqueous solution containing heparin in an amount sufficient to provide a cryoprecipitate/heparin solution containing from about 30 to about 150 units of heparin per milliliter of solution; (c) adding a sufficient amount of a precipitant consisting essentially of PEG to the cryoprecipitate/heparin solution while maintaining the solution at a temperature of from 20° C. to 30° C. to precipitate protein contaminants, leaving a PEG supernatant containing Factor VIII; (d) recovering the PEG supernatant; and (e) recovering Factor VIII from the PEG supernatant.

[0368] U.S. Pat. No. Re. 29,698 describes a method for improving the yield of AHF obtained from blood plasma and blood plasma fractions, obtained by cryoprecipitation, by the addition of heparin. The heparin-treated cryoprecipitate may then be further fractionated using polyethylene glycol and glycine. When the heparin-treated cryoprecipitate is further fractionated, heparin is preferably added twice, once to the initial cryoprecipitate and subsequently to the further fractionated concentrate.

[0369] (4) Preparation of Fraction Containing Soluble IL-1 Receptor or Soluble TNF Receptor

[0370] In one specific embodiment, the plasma is further separated into a fraction containing soluble IL-1 receptor or soluble TNF receptor. The preparation can be monitored by assaying for the physical properties of the receptors such as molecular weight, polarity, ionic strength, charge, isoelectric point, etc (Sambrook et al., *Molecular Cloning: A Laboratory Manual* (2nd Ed.), Cold Spring Harbor Laboratory Press, 1989). The preparation can also be monitored by assaying for the functional properties of the receptors such as the ability to specifically bind IL-1 or TNF, to block specific binding between IL-1 and IL-1 receptor or between TNF and a TNF receptor and to neutralize or reduce the biological activity of IL-1 or TNF. Preferably, the preparation is monitored by antibody-based assays and any anti-IL-1 soluble receptor and anti-TNF soluble receptor antibodies can be used (see Current Protocols in Immunology (Ed. Coligan et al.) John Wiley & Sons, Inc., 1997).

[0371] C. Methods of treatment using the resulting blood-derived compositions

[0372] The compositions thus produced are suitable for treating viral hemorrhagic diseases or disorders or other diseases, disorders or syndromes involving such cytotoxic responses including, but not limited to, other acute infectious diseases, sepsis, cachexia, rheumatoid arthritis and other autoimmune disorders, acute cardiovascular events, chronic myelogenous leukemia and transplanted bone marrow-induced graft-versus-host disease, septic shock, immune complex-induced colitis, cerebrospinal fluid inflammation, autoimmune disorders, multiple sclerosis. Accordingly, methods for treating or preventing a viral hemorrhagic disease or disorder or other such disorders involving such cytoxic responses in a mammal are provided. These methods include the steps of administering to the mammal an effective amount of the immune composition(s) produced according to the above processes.

[0373] Furthermore, such compositions can be used alone or in combination with a tetracycline or tetracycline-like compound(s) and/or other anti-viral-hemorrhagic agent(s), such as IL-1 inhibitors and TNF inhibitors. Any of the above noted disorders and disorders involving an acute inflammatory response can be treated by the compositions.

[0374] Viral hemorrhagic diseases can be treated by administration of tetracycline or tetracycline-like compound(s), The effectiveness of administration of a tetracycline compound or tetracycline-like compound(s) for treatment is optimal shortly after infection. Such treatment is preferably combined with administration of the compositions provided herein and/or other treatments for viral hemorrhagic disorders.

[0375] Methods for treating disorders involving acute inflammatory responses characterized by elevated and debilitating levels of cytokines are provided. These disorders include those enumerated herein and any others in which acute inflammatory responses, as assessed by elevated levels of TNF and/or IL-1, occur. Several methods are provided.

[0376] In one method a mammal determined to have an acute inflammatory response or a disease or condition characterized by such response is treated with a blood-derived composition provided herein. The mammal may also be treated with a tetracycline or tetracycline-like compound or plurality thereof and/or with a treatment known to have some effect on the symptoms of or on disorder. All treatments may be administered simultaneously, successively or intermittently and, as necessary, repeatedly and for a time sufficient to observe an amelioration or treatment of the symptoms of the disease, condition or disorder.

[0377] Hence, including among the methods provided herein, are methods in which such mammals are treated with blood or fraction thereof that has been contacted with a tetracycline or tetracycline-like compounds either in vitro or in vivo. Where the blood is treated in vivo, it is obtained from a donor who has been administered a tetracycline and tetracycline-like compounds prior to providing blood. Where the blood or a fraction thereof, particularly white blood cell-containing fraction, such as buffy coats, has been treated in vitro with a tetracycline and/or tetracycline-like compound(s), it is obtained from an untreated donor and then either fractionated prior to contacting or subsequent to contacting. In one embodiment, the blood is treated to obtain the buffy coat, which contains the white blood cells. The buffy coat fraction is contacted in vitro with a tetracycline and/or tetracycline-like compound(s). The medium from the treated cells is administered. It can be further fractionated or concentrated prior to administration. In all instances, the levels of the TNF and IL-1 receptors are monitored prior to contacting with the tetracycline and/or tetracycline-like compound(s), during and after contacting for at least a three-fold increase in the level of such receptors compared to the baseline, prior to contacting with the tetracycline and/or tetracycline-like compound(s). Such measure serves as indicator that the factors, which include sTNF receptors and/or IL-1 receptors, particularly IL-1 RA, have reached a sufficient level. These receptors serve as the marker for a sufficient level of induction of the palliative factors; they are not necessarily the only factors responsible for the observed effects.

[0378] These methods may also be combined with other methods for treating such disorders, such as other anti-IL-1 antibodies, anti-IL-1 receptor antibodies, IL-1 receptor antagonists, IL-1 production inhibitors, IL-1 receptor production inhibitors, and IL-1 releasing inhibitors.

[0379] Administration is effected by any suitable route, including systemic, local and topical administration, such as intramuscularly, intravenously, parenterally and orally. Typically, administration of a blood product will be via IV route. Administration of a tetracycline compound will be orally. Amounts of tetracycline is about 100-500 mg twice per day for one or more days, typically at least three and up to about ten days. These amounts are also the amounts for administration human donors to induce factors for preparation of the blood-derived compositions.

[0380] The disorders include hemorrhagic diseases and disorders, wasting diseases, sepsis, autoimmune disorders, particularly acute episodes associated with autoimmune disorders, acute episodes associated with multiple sclerosis, acute allergic reactions and other inflammatory diseases. The methods herein are particularly useful for treating hemorrhagic diseases or disorders, for which there have heretofore been few, if any, effective treatments.

[0381] In one method, a mammal suffering from such disorder is treated with an amount of a tetracycline and tetracycline-like compounds effective to ameliorate a symptom of the disorder, particularly, a disorder associated with elevated levels of cytokines associated with an acute inflammatory disorder. This method is intended for treatment of viral hemorrhagic fevers, and also bacterial infections, such as *E. coli* infections.

[0382] In another embodiment, the anti-viral-hemorrhagic agent is a tumor necrosis factor (TNF) inhibitor, including an anti-TNF antibody, an anti-TNF receptor antibody, a TNF receptor antagonist, a TNF production inhibitor, a TNF receptor production inhibitor or a TNF releasing inhibitor. In another exemplary embodiment, the anti-viral-hemorrhagic agent is an anti-viral vaccine, an anti-viral antibody, a viral-activated immune cell or a viral-activated immune serum. Any specific examples of the IL-1 inhibitor, the TNF inhibitor, the anti-viral vaccines, the anti-viral antibodies, the viral-activated immune cells or the viral-activated serum can be used in the combinational therapy.

[0383] The tetracycline compound(s) and/or the anti-viral-hemorrhagic agent(s) can be used alone or in combination with other known therapeutic agents or techniques (including chemotherapeutics, radioprotectants and radiotherapeutics) to either improve the quality of life of the patient, or to treat the disease, such as viral hemorrhagic diseases or disorders. For example, the tetracycline compound(s) and/or the anti-viral-hemorrhagic agent(s) can be used before, during or after radiation treatment.

[0384] F. Viral Hemorrhagic Diseases or Disorders and Diagnosis Thereof

[0385] The methods and compositions provided herein are particularly suited for treatment of viral hemorrhagic diseases. To effectively employ such methods, proper diagnosis is recommended. Hence following is a list of exemplary hemorrhagic diseases, the causative agents and methods of diagnosis.

[0386] Examples of the viral hemorrhagic diseases or disorders that can be treated by the present methods include, but not limited to, viral hemorrhagic disease caused by infection with Bunyaviridae, a Filoviridae, a Flaviviridae, or an Arenaviridae virus.

[0387] 1. Bunyaviridae Virus Infection

[0388] Examples of Bunyaviridae viruses include bunyavirus (Bunyamwera, Bwamba, California, Capim, Guama, phlebovirus koongol, patois, simbu and tete viruses), sandfly fever virus, Rift Valley fever virus of sheep and ruminants, Nairovirus, Crimean-Congo hemorrhagic fever virus, Uukuvirus, Uukuniemi virus, Hantaan virus and Korean hemorrhagic fever virus (see, e.g., U.S. Pat. No. 5,786,342). Of particular interest is treatment of Crimean-Congo hemorrhagic fever virus, Hantaan virus and Korean hemorrhagic fever virus infections, particularly, Hantaan virus. Specific strains of Hantaan virus include 76-118 strain (Avsic-Zupanc, et al., *Am. J. Trop. Med. Hyg.,* 51(4):393-400 (1994); Gu, et al., *Chin. Med. J. (Engl).,* 103(6):455-9 (1990); Miyamoto, et al., *Kansenshogaku Zasshi.,* 61f(6):633-8 (1987 Jun); and Miyamoto, et al., *Kansenshogaku Zasshi.,* 61(6):639-44 (1987 Jun)) and WKM strain (Yoo, et al., *Microbiol. Immunol.,* 37(7):557-62 (1993); and Yoshimatsu, et al., *J. Gen. Virol.,* 77(4):695-704 (1996 Apr)).

[0389] Bunyaviridae virus infection, and particularly Hantaan virus infection, can be diagnosed by any methods known in the art according to clinical, immunological or molecular criteria. Any known immunological methods can be used in the diagnosis of Bunyaviridae or Hantaan virus infection (see e.g., Current Protocols in Immunology (Ed. Coligan et al.) John Wiley & Sons, Inc., 1997); Sambrook et al., *Molecular Cloning: A Laboratory Manual* (2nd Ed.), Cold Spring Harbor Laboratory Press, 1989)). Such methods are known (see, e.g., Burkhardt, et al., *Fortschr. Med.,* 111 (33):528-9 (1993) and van Ypersele de Strihou, et al., *Lancet,* 2(8365-66):1493 (1983)). Antibody-based or antigen-based immunological methods include immunoprecipitation, Western blotting, dot blotting and in situ immunodetection methods such as immunofluorescence can be used. In a specific embodiment, anti-Bunyaviridae virus or anti-Hantaan virus antibodies described herein can be used in the immunodiagnosis.

[0390] Nucleotide-sequence based molecular methods include nucleotide sequencing, nucleotide hybridization, polymerase chain reaction (PCR), especially reverse-transcriptase polymerase chain reaction (RT-PCR) can be used. Hantaan virus nucleotide fragments with all or portions of the following Genbank Accession Nos. can be used in the nucleotide-sequence based molecular diagnosing methods: AF035831, X95077, D25531, D25528-D25530, D25532-D25533, U71369-U71372, U71281-U71283, X55901, S74081, S67430, U38911, U38910, Y00386, U38177, U37768, U37729, M14626, M57637, M14627, M57432 and L08753.

[0391] 2. Filoviridae Virus Infection

[0392] Filoviruses are classified in the order Mononegavirales (Pringle C. R., *Arch. Virol.,* 117:137-140 (1991)), which also contains the nonsegmented negative-strand RNA virus families Paramyxoviradae, Rhabdoviridae, and Bornaviridae. Members of the family Filoviridae includes Marburg virus, a unique agent without known subtypes, and Ebola virus, which has four subtypes (Zaire, Sudan, Reston, and Ivory Coast) (Feldmann and Slenczka Klenk, *Arch. Virol.* 11 (Suppl):77-100 (1996); LeGuenno B., et al., *Lancet,* 345:1271-127 (1995); Pringle C. R., *Arch. Virol.,* 117:137-140 (1991)). Specific strains of ebola virus include Zaire strain (Jaax, et al., *Lancet,* 346(8991-8992):1669-71 (1995), Andromeda strain (Johnson, *Ann. Intern. Med.,* 91(1):117-9 (1979), Gabon 94 strain (Prehaud, et al., *J. Gen. Virol.,* 79(11):2565-72 (1998) and Sudan, Reston, and Ivory Coast strains (Feldmann and Slenczka Klenk, *Arch. Virol.* 11 (Suppl):77-100 (1996); LeGuenno B., et al., *Lancet,* 345:1271-127 (1995); Pringle C. R., *Arch. Virol.,* 117:137-140 (1991)).

[0393] Filoviruses are enveloped, nonsegmented negative-stranded RNA viruses. The two species, Marburg and Ebola virus, are serologically, biochemically, and genetically distinct. Classification, virion morphology and structure, genomic organization and diagnosis are described in detail in Beer et al., *Naturwissenschaften,* 86:8-17 (1999), Springer-Verlag 1999. Marburg and Ebola viruses are pleomorphic particles that vary greatly in length, but the unit length associated with peak infectivity is 790 nm for Marburg virus and 970 nm for Ebola virus (Regnery et al., *J. Virol.,* 36:465-469 (1980)). The virions appear as either long filamentous (and sometimes branched) forms or in shorter

U-shaped, 6-shaped (mace-shaped), or circular (ring) configurations (Murphy et al., Paltyn S. R. (ed) Ebola virus hemorrhagic fever, Elsevier/North-Holland, Amsterdam, pp. 61-82 (1978); Peters et al., Martini and Siegert (eds) Marburg virus disease, Springer, Berlin Heidelberg, New York, pp. 68-83 (1971)). Virions have a uniform diameter of 80 nm and a density of 1.14 g/ml. They are composed of a helical nucleocapsid, a closely apposed envelope derived from the host cell plasma membrane, and a surface projection layer composed of trimers of viral glycoportein (GP) (Feldmann et al. (1991) *Virology* 182:353-356). All filoviruses contain one molecule of noninfectious, linear, negative-sense, single-stranded RNA with a M_r of 4.2×10^6, constituting 1.1% of the virion mass (Kiley M. P et al. (1988) *J. Gen. Virol.* 69:1957-1567 (1988); Regnery et al. (1980) *J. Virol.* 36:465-469).

[0394] The nonsegmented negative-strand RNA genomes of filoviruses show the gene arrangement 3'-NP-VP35-VP40-GP-VP30-VP24-L-5' with a total molecular length of approximately 19 kb (Table 2).

TABLE 2

Filoviral proteins and functions

Designation	Virus type	Encoding gene	Localization	Function
NP	MBG/EBO	1	Ribonucleocapsid complex	Encapsidation
VP35	MBG/EBO	2	Ribonucleocapsid complex	Phosphoprotein analogue
VP40	MBG/EBO	3	Membrane-association	Matrix protein
GP	MBG/EBO	4	Surface (transmembrane protein)	Receptor binding, fusion
VP30	MBG/EBO	5	Ribonucleocapsid complex	Encapsidation, necessary for transcription and replication
VP24	MBG/EBO	6	Membrane-association	Unknown (minor matrix protein, uncoating)
L	MBG/EBO	7	Ribonucleocapsid complex	RNA-dependent
sGP	EBO	4	Nonstructural, secreted	Unknown

NP nucleoprotein; VP virion structural protein; GP glycoprotein; L large protein (polymerase); sGP small glycoprotein; MBG type Marburg filoviruses; EBO type Ebola filoviruses Modified after Feldmann et al., Archives of Virology, 1996.

[0395] Filoviridae virus infection, and particularly ebola and Marburg virus infection, can be diagnosed by any methods known in the art according to clinical, immunological or molecular criteria (see, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual* (2nd Ed.), Cold Spring Harbor Laboratory Press, 1989). Antibody-based or antigen-based immunological methods include immunoprecipitation, Western blotting, dot blotting and in situ immunodetection methods such as immunofluorescence can be used. In a specific embodiment, anti-Filoviridae virus or anti-ebola and anti-Marburg virus antibodies, such as those described herein, can be used in the diagnosis of Bunyaviridae or Hantaan virus infection (see, e.g., Current Protocols in Immunology (Ed. Coligan et al.) John Wiley & Sons, Inc., 1997).

[0396] Nucleotide-sequence based molecular methods include nucleotide sequencing, nucleotide hybridization, polymerase chain reaction (PCR), especially reverse-transcriptase polymerase chain reaction (RT-PCR) can be used. In a specific embodiment, the ebola virus nucleotide sequences with the following Genbank Accession Nos. can be used in the nucleotide-sequence based molecular diagnosing methods: AF086833, U77384-U77385, U8116-U23417, U23187, U23152, U23069, AF034645, AF054908, X67110, L 11365, U28077, U28134, U28006, U31033, U23458, X61274, J04337 and M33062. In another specific embodiment, the Marburg virus nucleotide sequences with the following Genbank Accession Nos. can be used in the nucleotide-sequence based molecular diagnosing methods: AF005730-AF005735, Z12132, Z29337, X64405-X64406, X68493-X68495, M72714, M92834 and M36065.

[0397] Reverse transcriptase polymerase chain reaction is one of the most powerful tools of diagnosis of filovirus infection (Volchkov V., et al., *Virology*, 232:139-144 (1997)). Antibodies to filovirus can be detected by immunofluorescence assays using acetone-fixed virus-infected cells inactivated by A-radiation [Johnson et al., *Trans. R. Soc. Trop. Med. Hyg.*, 76:307-310 (1982); Johnson et al., *Trans. R. Soc. Trop. Med. Hyg.*, 77:731-733 (1983)), which should not be used under field conditions. An enzyme-linked immunosorbent assay using a mild detergent extract of infected Vero cells adsorbed to plastic plates has been shown to be more reliable (Ksiazek, *Lab. Anim.*, 20:34-46 (1991)) under such conditions.

[0398] Vero cells are readily used for the isolation and propagation of fresh and laboratory passaged strains of the viruses. MA-104 cells and SW13 cells have also been successful in primary filovirus isolation (McCormick et al., *J. Infect. Dis.*, 147:264-267 (1983)). In some circumstances primary isolation in guinea pigs (for Marburg virus) or suckling mice (for Ebola virus) may be required.

[0399] A western blot method has been standardized for the diagnosis of filovirus infections [Elliott et al., *J. Virol. Methods,* 43:85-89 (1993)). Solid-phase indirect enzyme-immunoassay (SPEIA) has been used to detect Lassa and Ebola virus antigens and antibodies using horseradish peroxidase-labeled antispecific globulins (Ivanov etal. (1985) *Vopr Virusol.* 31(2):186-190). Immunohistochemistry (IHC) testing of formalin-fixed postmortem skin specimens can also be performed (see, e.g., Zaki et al. (1999) *J. Infect. Dis.* 179(*Suppl1*):S36-47).

[0400] 3. Flaviviridae Virus Infection

[0401] All members of the Flaviviridae family share common morphologic characteristics, genome structure, and replication and translation strategies (see, e.g., Kautner, et al., *J. Pediatr.,* 131:516-524 (1997)). Examples of Flaviviridae viruses include flavivirus, Brazilian encephalitis virus, Bussuquara virus, Dengue virus, iiheus virus, Israel turkey meningoencephalitis virus, Japanese B encephalitis virus, Kunjin virus, Kyasanur forest disease virus, Langat virus, Louping ill virus, Modoc virus, Murray valley encephalitis virus, Ntaya virus, omsk hemorrhagic fever virus, powassan virus, St. Louis encephalitis virus, spondwnei virus, tick-borne encephalitis, Uganda S virus, US bat salivary gland virus, wesselsbron virus, West Nile fever virus, yellow fever virus, Zika virus, European tick-borne encephalitis, Far Eastern tick-borne encephalitis virus and Russian tick-borne encephalitis (U.S. Pat. No. 5,786,342). Preferably, the Dengue virus to be treated is a Dengue type 1, Dengue type 2, Dengue type 3 or Dengue type 4 virus. Specific Dengue type 1 virus strains include Singapore strain S275/90 (Fu, et al., *Virology,* 188(2):953-8 (1992)), Western Pacific strain (Puri, et al., *Virus Genes,* 17(1):85-8 (1998)) and Mochizuki strain (Zulkarnain, et al., *Micobiol. Immunol.,* 38(7):581-5 (1994)). Specific Dengue type 2 virus strains include Brazilian strain (Barth, et al., *Mem. Inst. Oswaldo. Cruz.,* 86(1):123-4 (1991)), New Guinea C strain (Biedrzycka, et al., *J. Gen. Virol.,* 68(5):1317-26 (1987); Irie, et al., *Gene,* 75(2):197-211 (189); Kapoor, et al., *Gene,* 162(2):175-80 (1995); Price, et al., *Am. J. Trop. Med. Hyg.,* 22(1):92-9 (1973)), strain 16681 (Kinney, et al., *Virology,* 230(2):300-8 (1997)), strain PR-159 (Leblois, et al., *Nucleic Acids Res.,* 21(7):1668 (1993)), Cuban A15 strain (Pupo-Antunez, et al., *Hybridoma.,* 16(4):347-53 (1997)) and Mexican strain (Sanchez, et al., *J. Gen. Virol.,* 77(10):2541-5 (1996)). Hence, the family Flaviviridae includes human pathogens, Dengue viruses, the Japanese encephalitis virus and yellow fever virus.

[0402] Four Dengue virus serotypes and various "biotypes" can be differentiated. Mature Dengue virus particles have a single-stranded ribonucleic acid genome surrounded by an approximately icosahedral nucleocapsid with a diameter of 30 nm. The nucleocapsid is covered by a lipid envelope of 10 nm thickness derived from host cell membranes and contains the envelope and membrane proteins (Westaway et al., *Flaviridiac. Intervirology,* 24:183-92 (1985)).

[0403] The viral genome of approximately 11 kb is infectious, has a messenger-like positive polarity, and can be translated in vitro. The 5' end of the RNA has a type I cap structure but lacks a poly A tail at the 3' end (Rice et al., *Science,* 229:726-33 (1985); Hahnet al., *Virology,* 162:167-80 (1988); Irie et al., *Gene,* 74:197-211 (1989)). It contains a single open reading frame of about 10,000 nucleotides encoding three structural and seven nonstructural proteins. The gene order is 5'-C-prM(M)-E-NSI-NS2A-NS2B-NS3-NS4A-NS4B-NS5. The proteins are synthesized as a polyprotein of about 3000 aminoacids that is processed cotranslationally and posttranslationally by viral and host proteases (Biedrzycka et al., *J. Gen. Virol.,* 1987, 68:1317-26; Mackow et al., *J. Gen. Virol.,* 1987, 69:23-4; Speight et al., *Virology,* 1987, 159(2):217-28; Chambers et al., *Virology,* 1989, 169:100-9; Markoff etal., *J. Virol.,* 1989, 63:3345-52; Preugschar et al., *J. Virol.,* 1990, 64:4364-74; Falgout et al., *J. Virol.,* 1991, 65:2467-75; Preugschat et al., *J. Virol.,* 1991, 65:4749-58; Preugschat F., et al., *Virology,* 1991, 185:689-97; Cahour et al., *J. Virol.,* 1992, 66:1535-42).

[0404] The structural proteins include a capsid protein rich in arginine and lysine residues and a nonglycosylated prM protein produced from a glycosylated precursor in a late step of virus maturation (Rice et al., *Science,* 1985, 229:726-33; Hahn et al., *Virology* 1988, 162:167-80; Deubel et al., *J. Virol. Methods,* 1990, 30:41-54;Randolph et al., *Virology* 1990, 174:450-8). The major structural envelope protein is involved in the main biologic functions of the virus particle such as cell tropism, acid-catalyzed membrane fusion, and the induction of hemagglutination-inhibiting, neutralizing, and protective antibodies (Depres et al., *Virology,* 1993, 196:209-219).

[0405] The first nonstructural protein is NSI, a glycoprotein with a function in the virus life cycle that is unknown (Schlesinger et al., *J. Immunol.,* 1985, 135:2805-9). NS1 proteins are detected in high titers in patients with secondary Dengue infections, but are rarely found in primary infections (Kuno et al., *J. Med. Virol.,* 1990, 32:102-8). The NS2 region codes for two proteins (NS2A and NS2B) that are thought to be implicated in polyprotein processing, whereas NS3 is probably the viral proteinase that functions in the cytosol (Preugschat et al., *Virology,* 1991, 185:689-97; Cahour et al., *J. Virol.,* 1992, 66:1535-42; Falgout et al., *J. Virol.,* 1989, 63:1852-60). The NS4 region codes for two small hydrophobic proteins that seem to be involved in the establishment of the membrane bound RNA replication complex. The protein encoded by the NS5 gene has a molecular weight of 105,000, is the most conserved flavivirus protein and is the virus-encoded RNA-dependent RNA polymerase.

[0406] Flaviviridae virus infection, and particularly Dengue virus infection, can be diagnosed by any methods known in the art according to clinical, immunological or molecular criteria. Any known immunological methods can be used in the diagnosis of Flaviviridae or Dengue virus infection (see Current Protocols in Immunology (Ed. Coligan et al.) John Wiley & Sons, Inc., 1997). Antibody-based or antigen-based immunological methods include immunoprecipitation, Western blotting, dot blotting and in situ immuno-detection methods such as immunofluorescence can be used. Antibodies described herein can be used in the immunodiagnosis.

[0407] Any known molecular methods can be used in the diagnosis of Flaviviridae or Dengue infection (Sambrook et al., *Molecular Cloning: A Laboratory Manual* (2nd Ed.), Cold Spring Harbor Laboratory Press, 1989). Nucleotide-sequence based molecular methods include nucleotide sequencing, nucleotide hybridization, polymerase chain reaction (PCR), especially reverse-transcriptase polymerase

chain reaction (RT-PCR) can be used. Dengue virus nucleotide fragments containing all or portions of sequences with the following Genbank Accession Nos. can be used in the nucleotide-sequence based molecular diagnosing methods: E06832, D10514, D10513, X70952.

[0408] The diagnosis of Dengue relies in most case on clinical judgment because only a few major centers have the facilities and means to perform and verify the clinical impression. Diagnostic criteria for DHS based on clinical observations have been proposed by the World Health Organization and should be used to avoid over-diagnosis (World Health Organization. Dengue hemorrhagic fever: diagnosis, treatment and control, Geneva, WHO, 1986). Clinical criteria for diagnosis are as follows: (1) fever; (2) hemorrhagic manifestations, including at least a positive tourniquet test result and a major or minor bleeding phenomenon; (3) hepatic enlargement; (4) shock (high pulse rate and narrowing of the pulse pressure to 20 mm Hg or less, or hypotension). The laboratory criteria include (5) thrombocytopenia ($\leq$100,000/mm^3), and (6) hemoconcentration (hematocrit increase $\geq$20%). Thrombocytopenia with concurrent high hematocrit levels differentiates DHF from classic DF.

[0409] A secondary Dengue infection is characterized by the rapid appearance of broadly cross-reactive antibodies. Hemagglutination inhibition titers of 1:20 in the acute-phase sample rise to $\geq$1:2560 in the convalescent phase sample. An antibody titer of $\geq$1:1280 in the acute-phase sample without a fourfold or greater increase in the second sample also is considered presumptive of recent infection. A less time-consuming method is a capture enzyme-linked immunosorbent assay that can detect specific anti-Dengue IgM in a single acute-phase sample (Lam et al., *Southeast Asian, J. Trop. Med. Public Health,* 1987, 18:532-8).

[0410] Commercial kits for the detection of specific IgG as well as IgM antibodies have become available. They are based on a dot enzyme assay or a nitrocellulose membrane-based capture format, respectively, and should be suitable for field research (Cardosa et al., *J. Virol. Methods,* 1988, 22:81-8; Cardosa et al., *Southeast Asian, J. Trop. Med. Public Health,* 1988, 19:591-4; Cardosa et al., *Clin. Diagn. Virol.,* 1995, 3:343-50).

[0411] An alternative to virus isolation is the detection of viral RNA by reverse transcription polymerase chain reaction. There are various protocols available using different primers and template isolation (Deubel et al., *J. Virol. Methods,* 1990, 30:41-54; Henchal et al., *Am. J. Trop. Med. Hyg.,* 1991, 45:418-28; Morita et al., *J. Clin. Microbiol.,* 1991, 29:2107-10; Morita et al., *J. Med. Virol.,* 1994, 44:54-8; Lanciotti et al., *J. Clin. Microbiol.,* 1992, 30:545-51; Suk-Yin et al., *Southeast Asian, J. Trop. Med. Public Health,* 1994, 25:258-61; Seah et al., *J. Virol. Methods,* 1995, 51:193-200). Reverse transcription polymerase chain reaction coupled with hybridization with labeled serotype-specific probes can detect as few as 4 plaque-forming units per 100 μl serum and gives the best results early in the acute phase of the disease when Dengue antibodies are still low (Suk-Yin et al., *Southeast Asian, J. Trop. Med. Public Health,* 1994, 25:258-61). Less than 1 μl of serum can be sufficient for the detection of viral RNA (Chan et al., *J. Virol. Methods,* 1994, 49:315-22).

[0412] 4. Arenaviridae Virus Infection

[0413] Examples of Arenaviridae viruses include Junin virus, Lassa virus, Machupo virus, Pichinde virus, lymphocytic choriomeningitis virus, Lassa fever virus and arenavirus (U.S. Pat. No. 5,786,342). Preferably, the Arenaviridae viruses to be treated are Junin virus, Lassa virus, Machupo virus. Specific strains of Lassa virus include Josiah strain (Auperin, et al., *Virology,* 168(2):421-5 (1989); and Fidarov, et al., *Vopr Virusol.,* 35(4):326-9 (1990) and Nigerian strain (Clegg, et al., *Virus Res.,* 18(2-3):151-64 (1991)).

[0414] Arenaviridae virus infection, and particularly Lassa virus, Machupo virus, or Pichinde virus infection, can be diagnosed by any methods known in the art according to clinical, immunological or molecular criteria. Any known immunological methods can be used in the diagnosis of Arenaviridae virus infection, and particularly Lassa virus, Machupo virus, or Pichinde virus infection (see Current Protocols in Immunology (Ed. Coligan et al.) John Wiley & Sons, Inc., 1997). Antibody-based or antigen-based immunological methods include immuniprecipitation, Western blotting, dot blotting and in situ immuno-detection methods such as immunofluorescence can be used. In a specific embodiment, anti-Arenaviridae virus or anti-Lassa virus, anti-Machupo virus and anti-Pichinde virus antibodies known to those of skill art in the or described herein can be used in the immunodiagnosis.

[0415] Any known molecular methods can be used in the diagnosis of Arenaviridae virus infection, and particularly Lassa virus, Machupo virus, or Pichinde virus infection (Sambrook et al., *Molecular Cloning: A Laboratory Manual* (2nd Ed.), Cold Spring Harbor Laboratory Press, 1989); see also, Sarrat, et al., *Bull Soc Pathol Exot Filiales.,* 65(5):642-50 (1972) (Histopathological diagnosis of hepatitis due to Lassa virus); and Trappier, et al., *Am. J. Trop. Med. Hyg.,* 49(2):214-21 (1993) (Evaluation of the polymerase chain reaction for diagnosis of Lassa virus infection)).

[0416] Nucleotide-sequence based molecular methods include nucleotide sequencing, nucleotide hybridization, polymerase chain reaction (PCR), especially reverse-transcriptase polymerase chain reaction (RT-PCR) can be used. Lassa virus nucleic acid fragments containing sequences from the following Genbank Accession Nos. can be used in the nucleotide-sequence based molecular diagnosing methods: U80004, U73034-U73035, U63094, X52400, J04324, K03362 and M15076. Machupo virus nucleic acid fragments containing sequences from the following Genbank Accession Nos. can be used in the nucleotide-sequence based molecular diagnosing methods: X62616.

[0417] G. Examples

[0418] The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLE 1

Treatment of Marburg and Lassa Virus Infection

[0419] a. Experimental Protocals

[0420] (1) Virus

[0421] Marburg virus strain Popp used in the following experiments was received from the Belarussian Research Institute of Epidemiology and Microbiology (Minsk,

Belarussia). All work with infectious virus was performed in the maximum-containment biosafety level-4 (BSL-4) facility of the State Scientific Center of Virology and Biotechnology ("Vector") (Koltsovo, Russia). This virus was amplified in Vero E 6 cells and the supernatant was collected to produce stocks. This stock virus suspension (2×10^7 PFU/ml) was stored at −70° C.

[0422] Lassa virus strain Josiah used in the following experiments was received from Belarussian Research Institute of Epidemiology and Microbiology (Minsk, Belarussia). This mouse-adapted Lassa virus was passaged once in Vero E6 cells and 3 times passaged in mice by intracerebrally challenge. This mouse-adapted Lassa virus stock was collected and stored at −70° C. This stock contained 10^6 PFU ml (or 10^5 LD_{50} by inoculation challenge of 4-week old BALB/c mice).

[0423] (2) Animals

[0424] Outbred Hartly guinea-pigs of 200-220 grams were used in the experiments with Marburg virus. Four-week old BALB/c mice (haplotype H-2d) were used in the experiments with Lassa virus.

[0425] The animals were received from the vivarium of SRC VB "Vector" and kept at a standard ration. To ensure that the animals (guinea-pigs and mice) were spared of unnecessary pain and discomfort, standard anesthesia methods were used. A single dose of ketamine/xylazine via intramuscular injection in the posterior region of the hind leg was administered to the animals.

[0426] (3) PCR

[0427] RT-PCR procedure for Lassa virus detection was performed as described in Demby et al., *J. Clinical Microbiology,* 32:2898-2903 (1994) and for Marburg virus detection as described in Ignatyev et al., In: Berg D. A. (ed) Proceedings of the 1996 ERDEC scientific conference on chemical and biological defense research, Nov. 19-22, 1996, pp. 323-330 (1996).

[0428] b. Treatment of Marburg Virus Infection

[0429] Animals were divided into 11 groups, each containing 6 animals:

[0430] 1. Animals of the first group serve as virus controls, ie., were infected with the virus but were not given therapeutic or prophylactic or any treatment agents.

[0431] 2_t. Animals of the second group (T) were given 1 ml of Tetracycline-Hcl (Belmedpreparats Ltd., Russia) solution (58 mg/kg) intramuscularly from 10 days before virus injection until seventh day after virus injection daily.

[0432] 2_d. Animals of the second group (D) were given 1 ml of Doxycycline solution (Belmedpreparats Ltd., Russia) (58 mg/kg) intramuscularly from 10 days before virus injection until seventh day after injection daily.

[0433] 3_t. Animals of the third group (T) were given 1 ml of Tetracycline-Hcl solution (58 mg/kg) intramuscularly from 5 days before virus injection until seventh day after injection daily.

[0434] 3_d. Animals of the third group (D) were given 1 ml Doxycycline solution (58 mg/kg) intramuscularly from 5 days before virus injection until seventh day after injection daily.

[0435] 4_t. Animals of the fourth group (T) were given 1 ml of Tetracycline-Hcl solution (58 mg/kg) intramuscularly from the third day after virus injection until seventh day after virus injection daily.

[0436] 4_d. Animals of the fourth group (d) were given 1 ml Doxycycline solution (58 mg/kg) intramuscularly from the third day after virus injection until seventh day after virus injection daily.

[0437] 5_t. Animals of the fifth group (T) serve as the Tetracycline controls, i.e., were given Tetracycline-Hcl solution (58 mg/kg) intramuscularly during the 17 day period without virus injection.

[0438] 5_d. Animals of the fifth group (d) serve as the Doxycycline controls, i.e., were given Doxycycline solution (58 mg/kg) intramuscularly during the 17 day period without virus injection.

[0439] Animals of the above groups were parenterally infected with Marburg virus at a dose of $5LD_{50}$ on day "0". The virus was detected by RT-PCR on the third day after infection.

[0440] As seen in Table 3, tetracycline and doxycycline are not toxic to control groups (5T, 5D). Using tetracycline and doxycycline prophylactically does not improve survival rate of the animals (2T, 2D, 3T and 3D). In fact, the mean time to death (m.t.d.) of these groups is shorter than that of the virus control group (1). In contrast, using tetracycline and doxycycline therapeutically increases survival rate of the animals because 2 animals from the group 4T and 4D, respectively, survived the otherwise lethal infection. In addition, the m.t.d. of groups 4T and 4D is slightly longer than that of the virus control group (1).

TABLE 3

Tetracycline and Doxycycline by experimental Marburg-virus infection

Group	guinea pigs (total)	survival	m.t.d.	
1	6	0	8.2	(control virus)
2T	6	0	8.06	
2D	6	0	7.69	
3T	6	0	7.91	
3D	6	0	7.6	
4T	6	2	8.75	
4D	6	2	8.54	
5T	6	6	—	(tetracycline control)
5D	6	6	—	(doxycycline control)

m.t.d. - mean time to death

[0441] m.t.d.—mean time to death

[0442] C. Treatment of Lassa Virus Infection

[0443] Animals were divided into the following groups, each containing 20 mice:

[0444] 1. Animals of the first group were infected with Lassa virus without any tetracycline or doxycycline treatment.

[0445] 2. Animals of the second group were given 0.2 ml of Tetracycline-HCl solution (58 mg/kg) from the third day until 7th day after virus injection (every day).

[0446] 3. Animals of the third group were given 0.2 ml of Doxycycline-HCl solution (58 mg/kg) form the third day until 7th day after virus injection (every day).

[0447] 4. Animals of the fourth group were given Tetracycline-HCl solution during a 7 day period without viral infection.

[0448] 5. Animals of the fifth group were given Doxycycline-HCl solution during a 7 day period without viral infection.

[0449] Animals of groups 1-3 were infected intracerebrally with Lassa virus at a dose of 10 PFU/0.03 ml on day "0". The virus was detected by RT-PCR on the third day after infection.

[0450] As seen in Table 4, tetracycline and doxycycline are not toxic to control groups (1). Using tetracycline and doxycycline therapeutically increases survival rate of the animals because Group 2 and 3 have higher survival rates than Group 1 (P<0.01). In addition, the m.t.d. of groups 2-3 is slightly longer than that of Group 1.

[0451] Levels of IL-1, IL-1Ra, TNF and soluble TNF receptor (sTNFR) were monitored in the Lassa virus control animals (Table 5) and tetracycline or doxycycline treated animals (Table 6) by ELISA using the ELISA kits or antibodies from R&D Systems, Inc. (U.S.A.). The ratio of IL-1/IL-1Ra in virus control animals (Table 5) increased dramatically to about 20 fold of the base level (Day 9) as the infection progressed and then returned to the base level (Day 21). In contrast, the ratio of IL-1/IL-1Ra in tetracycline or doxycycline treated animals (Table 6) increased to only about 5 fold of the base level (Day 3) and then returned to the base level (Day 21). Based upon the kinetics of the IL-1/IL-Ra ratio and sTNFr, treatment with a tetracycline compound appears to abort or limit infection.

TABLE 4

Tetracycline and Doxycycline for experimental Lassa - virus infection

Group	Total	Mice death	survival	m.t.d.
1 (virus control)	20	12	8	8.92
2 (doxycycline treatment)	20	6	14	9.09
3 (tetracycline treatment)	20	4	16	9.43
4 (doxycycline control)	20	0	20	n.d.
5 (tetracycline control)	20	0	20	n.d.

m.t.d. - mean time to death
n.d. - no detection

[0452] m.t.d.—mean time to death n.d.—no detection

TABLE 5

IL-1, IL-1Ra, TNF and sTNFr production in control animals CONTROL Lassa VIRUS (pg/ml) (Survival 8 from 20)

DAYS	IL-1	IL-IRA	IL-1/IL-IRA	TNF	sTNFr
0	1.9	51	0.037	1.56	12.6
1	7.6	66	0.115	4.8	16.4
3	21.84	120	0.182	22.6	25
5	41.5	130	0.319	22.8	25
7	47.88	121	0.395	23.4	25
9	49.92	66	0.756	22.6	25
m.t.d. 8.92					
15	22.15	121	0.183	16.4	100
21	3.2	63	0.050	2.4	18.2

[0453]

TABLE 6

IL-1, IL-1ra, TNF and sTNFr production in Lassa virus infected animals

	Doxycycline					Tetracycline				
DAYS	IL-1	IL-IRA	IL-1/IL-IRA	TNF	TNFR	IL-1	IL-IRA	IL-1/IL-IRA	TNF	sTNFR
0	1.9	51	0.037	1.56	12.6	1.9	51	0.037	1.56	12
1	7.6	66	0.115	4.8	16.4	7.6	66	0.115	4.8	16
3	21.84	120	0.182	22.6	25	21.84	120	0.182	22.6	2
5	38.3	280	0.136	20.4	52	19.4	180	0.107	26.4	40
7	31.2	500	0.060	17.16	751	12.48	200	0.062	20.28	100
9	16.6	690	0.024	16.2	721	10.2	520	0.019	17.2	120
15	12.48	175	0.073	14.04	20	7.8	84	0.091	14.82	50
21	2.6	56	0.046	2.1	13.8	2.4	54	0.044	2.0	16
	m.t.d. 9.09 survival 14 (20) 70%					m.t.d. 9.43 survival 16 (20) 80%				

EXAMPLE 2

Treatment of Dengue Virus Infection

[0454] a. Experimental Protocols

[0455] (1) Virus

[0456] Dengue virus, type 2 was used in the following experiments. All work with infectious virus was performed in the maximum-containment biosafety level-3 (BSL-3) facility of the "Vector". This virus was amplified in the brain of succlik mice (inbred BALB/c mice from Vector) and was collected to produce stocks. This stock virus suspension was stored at −40° C., containing 6.8 lg LD_{50}/ml (in mice BALB/c by intraperitoneal challenge).

[0457] (2) Animals

[0458] 4-week old BALB/c mice (haplotype H-2d) were used in the experiments with Dengue virus infection. Mice weigh 12-14 grams. The animals were received from SRC VB "Vector" and kept at a standard ration.

[0459] (3) RT-PCR Procedure

[0460] The virus detection was provided by PCR-method. Primers for Dengue virus type 2 detection are upper 5'AATATGCTGAAACGCGAGAGAAACCG (position 136-161 of the Dengue virus RNA SEQ ID No. 23 and lower 5'AAGGAACGCCACCAAGGCCATG (position 237-258) SEQ ID NO. 24.

[0461] RNA was extracted from serums of infected animals (mice) using the RNeasy Kit (Quigen, Germany). For RT-PCR, Titan kits (Behringer, Germany) were used. Reverse transcription was conducted at 42° C. for 60' followed by 40 amplification cycles at 94° C. for 30", 55° C. for 1', and 68° C. for 2' with a final extension at 68° C. for 7 mins. Amplification was conducted in 0.2 ml tubes with a model BIS-105M thermocycler (Russia).

[0462] b. Treatment of Dengue Virus Infection

[0463] Group 1

[0464] The animals of this group (60 animals) were given Doxycycline solution (58 mg/kg) intramuscularly every day for 4 days. From the first day, sera were taken from mice daily to detect concentration of IL-1, TNF, IL-1RA and sTNFr (Table 7).

[0465] Group 2

[0466] Animals of the second group are virus controls, i.e., were infected with the Dengue virus without doxycycline treatment. The virus detection was provided by PCR-method on the second day after infection. From the first day after infection, sera were taken from mice daily to detect concentration of IL-1, TNF, IL-1RA and sTNFr (Table 8).

[0467] Group 3

[0468] The animals from this group were given 0.2 ml of Doxycycline solution (58 mg/kg) intramuscularly from the second day after virus injection till the fifth day daily. The virus detection was provided by PCR-method on the second day after infection. From the first day after infection, sera were taken from mice daily to detect concentrations of IL-1, TNF, IL-1RA and sTNFr (Table 9).

[0469] Group 4

[0470] The animals from this group were given 0.2 ml of doxycycline solution (58 mg/kg) intramuscularly from the third day after virus injection till the fifth day daily. The virus detection was provided by PCR-method on the second day after the infection. From the first day after infection, sera were taken from mice daily to detect concentration of IL-1, TNF, IL-1RA and sTNFr (Table 10).

[0471] Group 5

[0472] The animals from this group were given, intravenously daily from the second day after infection till the sixth day, 0.3 ml of the serum collected from the animals of the group 1 on the first day after those animals were treated with doxycycline. In this volume, the Serum collected from the animals of group 1 contain 6.6 pg IL-1, 60 pg IL-1ra, 1.5 pg TNF and 25 pg sTNFr. The virus detection was provided by PCR-method on the second day after infection. From the first day after infection, the sera were taken from the mice of group 5 to detect concentration of IL-1, TNF, IL-1RA and sTNFr (Table 11).

[0473] Group 6

[0474] The animals from this group were given, intravenously daily from the second day after infection till the sixth day, 0.3 ml of the serum collected from the animals of the group 1 on the second day after those animals were treated with doxycycline. In this volume, the Serum collected from the animals of group 1 contain 6 pg IL-1, 20 pg IL-1ra, 5.5 pg TNF and 12 pg sTNFr. The virus detection was provided by PCR method on the second day after animals infection. From the first day after infection, sera were taken from the mice of Group 6 daily to detect concentration of IL-1, TNF, IL-1RA and sTNFr (Table 12).

[0475] C. Results and Discussion

[0476] As seen in Table 7, injection of doxycycline to the uninfected mice increases production of the cytokines and their soluble receptors. It is noteworthy that this response, i.e., increased production of cytokines and their soluble receptors, to the first doxycycline injection was higher than to the second and the third doxycycline injection. This difference signifies development of the refractory period in mice on the second and the third day after the injection of doxycycline. Therefore, multiple injections of doxycycline to the uninfected mice does not keep high concentrations of the soluble cytokine receptors in their sera. Also, the survival rate for group 5, which received serum containing 60 pg of IL-1Ra was higher than that in group 6 in which the IL-1Ra level was 20 pg.

[0477] The experiments using BALB/c (haplotype H-2d) and C57Bl/6 (H-2b) mice show that the dosage of Dengue virus of 10-10,000 LD_{50} is absolutely lethal (100%) after intraperitoneal challenge to these mice weighing 12-14 grams.

[0478] In the experiments described below, BALB/c mice weighing 12-14 grams were used. These mice died toward the end of the fifth day after the infection with the dose of Dengue virus 100 LD_{50}. In the sera of animals from Group

2 (virus control group), the concentration of IL-1 increases during the development of the infection more significantly than the concentration of IL-1RA (Table 8). The large excess of IL-1 over IL-1RA manifests in the ratio of IL-1/IL-1RA.

[0479] These experiments show the importance of detecting the ratio IL-1/IL-1RA in prognosis of the development of the disease caused by the Dengue virus infection. The change in the ratio of TNF to sTNFr during the course of Dengue virus infection is analogous to that of the ratio of IL-1 to IL-1RA. Overall, the concetration of these two cytokines increases more significantly than that of their respective receptors during the course of the infection. The concentration of TNF increased 500 times on the day of death but the concentration of sTNFr only increased 4 times. In addition, the ratio of TNF/sTNFr, rather than the TNF concentration itself, is more significant for the resolution of Dengue virus infection.

TABLE 7

Level of IL-1, TNF, soluble receptors: IL-IRA and sTNF after Doxycycline solution injection (Group 1)

Days	IL-1 (pg/ml)	IL-IRA (pg/ml)	IL-1/ IL-IRA (I)	TNF (pg/ml)	sTNFrI (pg/ml)	TNF/s TNFrI (II)	I + II
			Before the injection				
0	2.95	30	0.098	1.17	17	0.068	0.166
			After the injection				
1*	20.62	180	0.115	4.68	85	0.072	0.187
2**	17.43	60	0.291	17.55	38	0.462	0.753
3	17.48	80	0.219	9.36	35	0.267	0.486
4	17.93	175	0.102	8.19	38	0.216	0.318

*- Serum 1
**- Serum 2

[0480]

TABLE 8

Level IL-1, TNF, soluble receptors: IL-1RA and sTNF during of the experimental Dengue-virus infection (Group 2)

DAYS	IL-I (pg/ml)	IL-IRA (pg/ml)	IL-I/IL-IRA (I)	TNF (pg/ml)	sTNFrI (pg/ml)	TNF/sTNF (II)	I + II	Survival/ dead
0	2.95	30	0.098	1.17	17	0.068	0.166	10/0
1	10.6	70	0.151	8.19	32	0.256	0.407	10/0
2	16.8	65	0.258	26.9	37	0.727	0.985	10/0
3	26.7	70	0.381	35.1	45	0.780	1.161	10/0
4*	32.76	78	0.420	51.6	45	1.147	1.567	8/2
5*#	40.6	92	0.441	562.5	65	8.654	9.095	0/8
				m.t.d.-4.76				

*-blood samples taken from mice with clinical symptoms.
#-5 mice dies to the beginning of the fifth day and 3 mice to the end of this day.
m.t.d.-mean time of death

[0481]

TABLE 9

Level of IL-1, TNF, soluble receptors; IL-1RA and sTNF during the Doxycycline treatment (from the second day) of the experimental Dengue-infection (type 2) (Group 3)

Days	IL-1 (pg/ml)	IL-IRA (pg/ml)	IL-1/ IL-IRA (I)	TNF (pg/ml)	sTNFrI (pg/ml)	TNF/sTNF (II)	I + II	Survival/ dead
0	2.95	30	0.083	1.17	17	0.068	0.151	10/0
1	10.6	70	0.151	8.19	32	0.256	0.407	10/0
2	16.8	65	0.258	26.9	37	0.727	0.985	10/0
			the beginning of the treatment					
3	17.9	85	0.211	19.89	46	0.432	0.643	10/0
4	24.18	76	0.318	24.57	50	0.491	0.809	10/0
5	30.42	78	0.390	262.5	70	3.75	4.14	10/0
6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0/10

m.t.d.-mean time to death—6 days
n.d.-no death

[0482]

TABLE 10

Level of IL-1, TNF, soluble receptors; IL-1RA and sTNF during the Doxycycline treatment (from the third day) of the experimental Dengue (type 2) virus infection (Group 4)

Days	IL-1 (pg/ml)	IL-IRA (pg/ml)	IL-1/ IL-IRA (I)	TNF (pg/ml)	sTNFrI (pg/ml)	TNF/ sTNF (II)	I + II	Surviv-al/ dead
0	2.95	30	0.083	1.17	17	0.068	0.151	10/0
1	10.6	70	0.151	8.19	32	0.256	0.407	10/0
2	16.8	65	0.258	26.9	37	0.727	0.985	10/0
3	26.7	70	0.381	35.1	45	0.780	1.161	10/0
the beginning of the treatment								
4*	30.42	76	0.400	46.8	48	0.975	1.375	6/4
5#*	36.6	84	0.435	337.5	70	4.821	5.256	2/4 1/3
6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0/1

n.d.-no death

[0483]

TABLE 11

Level of IL-1, TNF, soluble receptors: IL-IRA and sTNF during the treatment by Serum N1 of the experimental Dengue (type 2) virus infection (Group 5)

Days	IL-1 (pg/ml)	IL-IRA (pg/ml)	IL-1/ IL-IRA (I)	TNF (pg/ml)	sTNFrI (pg/ml)	TNF/ sTNF (II)	I + II	Surviv-al/ dead
0	2.95	30	0.083	1.17	17	0.068	0.151	10/0
1	10.6	70	0.151	8.19	32	0.256	0.407	10/0
2	16.8	65	0.258	26.9	37	0.727	0.985	10/0
beginning of the treatment								
3	22.4	90	0.248	28.4	66	0.430	0.678	10/0
4	28.6	90	0.317	32.6	74	0.440	0.757	10/0
5	38.8	96	0.404	196.8	89	2.21	2.614	10/0
6	52.4	98	0.534	326.6	98	3.33	3.866	2/8
7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0/2

m.t.d.-mean time to death—6.21 days
n.d.-no death

[0484]

TABLE 12

Level of IL-1, TNF, soluble receptors: IL-IRA and sTNF during the treatment by serum N2 of the experimental Dengue (type 2) virus infection (Group 6)

Days	IL-I (pg/ml)	IL-IRA (pg/ml)	IL-1/ IL-IRA (I)	TNF (pg/ml)	sTNFrI (pg/ml)	TNF/ sTNF (II)	I + II	Surviv-al/ dead
0	2.95	30	0.083	1.17	17	0.068	0.151	10/0
1	10.6	70	0.151	8.19	32	0.256	0.407	10/0
2	16.8	65	0.258	26.9	37	0.727	0.985	10/0
the beginning of the treatment								
3	28.4	75	0.378	30.6	50	0.612	0.990	10/0
4	35.2	84	0.419	48.8	54	0.903	1.322	8/2
5	42.4	88	0.481	316.4	76	4.16	4.541	2/6
6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0/2

n.d.-no death

[0485]

TABLE 13

Effects of the different methods of treatment of the experimental Dengue (type 2) virus infection

Group	Scheme of Treatment	Survival/dead	m.t.d.
2	Virus control	0/10 2 mice - on 4 day 8 mice - on 5 day	4.76
3	doxycycline (from the 2 day till 5 day after infection)	0/10 10 mice on day 6	6.00
4	doxycycline (from the 3 day till 5 day after infection)	0/10 4 mice - on 4 day 5 mice - on 5 day 1 mice - on 6 day	4.62
5	serum 1 (from the 2 day till 5 day after infection)	0/10 8 mice - on 6 day 2 mice - on 7 day	6.21
6	serum 2 (from the 2 day till five day after infection)	0/10 2 mice - on 4 day 6 mice - on 5 day 2 mice - on 6 day	4.92

m.t.d. - mean time to death - 4.92 days

EXAMPLE 3

Treatment of Endotoxic Shock, Mousepox, Lassa Fever, Hemorrhagic Fever with Renal Syndrome (HFRS) and Dengue Fever with a Tetracycline Compound, IL-1Ra and Combinations Thereof

[0486] a. Expression of Soluble IL-1 Receptor Antagonist (IL-1Ra) in *E. coli*

[0487] The coding region of the IL-1Ra (residues 3-152, numbering according to Eisenberg et al. (1990) *Nature* 343:341-346; see, also Arend et al. (1990) *J.*

[0488] Clin. Invest. 85:1694-1797 and Hannum et al. (1990) *Nature* 343:336-340) as amplified from U937 cDNA by PCR with the introduction of an additional glycine residue, a BamHI restriction site at the 5' end and an EcoRi site at the 3' end (5' oligonucleotide CGG GAT CCG GGA GAA AAT CCA GCA AGA TG SEQ ID NO. 25; 3' oligonucleotide CGG AAT TCC CCT ACT CGT CCT GGA SEQ ID NO. 26). Using these primers, the mature recombinant IL-1Ra protein has the N-terminal sequence GSGRK, which is different from that of the native IL-1Ra protein, which is RPSGRK. The PCR product was introduced into the fusion protein expression vector pGEX-2T (Pharmacia; see, also Smith et al. (1988) Gene 67:21-40) and transformed into the *E. coli* strain NM554 (well known, see, e.g., Raleigh et al. (1988) *Nucl. Acids Res.* 16:1563-1575; and commercially available from, for example, Stratagene, La Jolla, Calif.). The expressed fusion protein glutathione S-transferase (GST)-IL-1Ra is cleaved with thrombin to obtain an authentic recombinant IL-1Ra protein.

[0489] b. Monitoring Production of TNF, Soluble TNF Receptor (sTNF R), IL-1, IL-1Ra in the Following Disease Models

[0490] There are disease models for monitoring disease progression and the efficacy of various treatment protocols. Exemplary models are as follows.

[0491] (1) Schwarzmann Reaction (Endotoxic Shock)

[0492] Endotoxic shock is accompanied by an increased IFN, TNF and IL-1 production, which simulates bacterial infection. BALB/c mice model are used in this study.

[0493] (2) Ectomelia (Mousepox)

[0494] BALB/c mice model are used in this study. Development of this lethal disease is accompanied by the increased TNF, IL-1 and IFN production.

[0495] Ectomelia virus gains entry through minute abrasions of the skin where it multiplies to produce a primary lesion. While this lesion is developing, a series of invasive steps produce a secondary viremia that seeds the skin and other organs with virus. A rash appears about 3 days after the primary lesion occurs.

[0496] (3) Experimental Lassa Fever

[0497] CBA/calac mice, which are highly sensitive to Lassa virus infection, are used in this study. Infection with the Lassa virus in the CBA/calac mice is accompanied by inflammation characterized histologically by cerebral edema, functional activity of kupffer cells, and necrosis of individual hepatocytes. Marked cytokine production also accompanies the disease development.

[0498] (4) Experimental HFRS Fever (Hantaan Virus)

[0499] C57B1/6 mice, which are highly sensitive to Hantaan virus infection, are used in this study. Development of this lethal disease is accompanied by the increased TNF and IL-1 production.

[0500] (5) Experimental Dengue Fever

[0501] BALB/c mice are used in this study. The mice are infected with denver fever virus. Development of this lethal disease is accompanied with by increased TNF, IL-1 and IFN production.

[0502] The data on dynamics of TNF, IL-1, sTNF and IL-1ra production and also dynamics of viremia are collected. These data allow the interrelationships between these cytokines, soluble receptors and the disease course to be determined. The scheme of administration of the soluble IL-1raand anti-TNF and anti-IL-1 drugs, which are likely to provide the healing of Systemic Inflammatory Response Syndrome (SIRS) in the above models, are based on the results thus obtained.

EXAMPLE 4

Treatment of the Dengue Virus Infection with Various Tetracycline and Tetracycline-like Compounds

[0503] Materials

[0504] Virus

[0505] Dengue virus type 2. Virus amplification by two passes through the brains of suckling mice. Mice were infected with 5 LD_{50}'s of virus.

[0506] Animals

[0507] 160 mice BALB/c (haplotype H-2d), age 4 weeks were used for the experiment.

[0508] Experimental Scheme

[0509] A groups, control groups (virus only; 50 mice) Group A1, 20 mice, was the control group for mortality.

[0510] Group A2, 30 mice, was used for obtaining blood samples on the day (0) and days 1, 3, 5 and 6 post infection. Blood samples were obtained from the orbital sinuses (at every time point 3 mice were used for harvesting blood). All blood samples (500 μl) were frozen (−70 C.). After completion of the experiment, the concentrations of TNF and IL-1 were measured.

[0511] B groups, 60 mice, treatment with tetracycline hydrochloride (20 mg/kg) from the third day before the virus infection until 8 days after virus injection administered twice per day, orally in a volume of 30 μl.

[0512] Group B1, 20 mice, control for mortality.

[0513] Group B2, 40 mice, was used to obtain blood samples on the day (−1), (0) and days 1, 3, 5, 6, 7 and 12 post infection. Blood samples were obtained from the orbital sinuses (at every time point 3 mice were used for harvesting blood). All blood samples (500 μl) were frozen (−70 C.). After completion of the experiment the concentrations of TNF, IL-1 were measured.

[0514] C groups, 60 mice, treatment with Vybromycine suspension (20 mg/kg) from the third day before the virus infection until 8 days after virus injection, twice per day, orally in a volume of 30 μl.

[0515] Group C1, 20 mice, control for mortality.

[0516] Group C2, 40 mice, was used to obtain blood samples on day (−1), (0) and days 1, 3, 5, 6, 7, 8 and 12 post infection. Blood samples were obtained from the orbital sinuses (on every time point 3 mice were used for harvesting blood). All blood samples (500 μl) were frozen (−70 C.). After the whole experiment had finished, the concentrations of TNF, IL-1 were measured.

[0517] D groups, 60 mice, treatment with Terramycine (20 mg/kg) from the third day before the virus infection until 8 days after virus injection, twice per day, intramuscularly in volume 100 μl.

[0518] Group D1, 20 mice, control for mortality.

[0519] Group D2, 40 mice, was used to obtain blood samples on day (−1), (0) and days 1, 3, 5, 6, 7, 8 and 12 post infection. Blood samples were obtained from the orbital sinuses (on every time point 3 mice were used for harvesting blood). All blood samples (500 μl) were frozen (−70 C.). After the whole experiment had finished, the concentrations of TNF, IL-1 were measured. On the third day after challenge by the Dengue virus all samples taken from the infected mice were tested by RT-PCR for the virus detection.

TABLE 13

Dynamics of the changes of the concentrations of TNF-α and IL-1β in the serum of animals from all Groups.

Group	Days	IL-1 pg/ml	TNF pg/ml	Survival/total	amount
Groups A					
A2 (virus control (30 mice)					Group A1 (20 mice) % (survival) 5
	0	7.0	18.4	20/20	m.t.d. = 5.5
	1	12.2	22.6	20/20	
	3	54.8	50.8	20/20	
	5	80.2	112.5	12/20	

TABLE 13-continued

6	166.8	136.6	4/20	
7	n.d	n.d	1/20	
12			1/20	
B2 (tetracycline treatment; 40 mice)				Group B1 (20 mice) % (survival) 40
-1	6.8	18.4	20/20	m.t.d. = 5.84
0	6.8	16.0	20/20	
1	10.8	16.6	20/20	
3	46.8	14.0	20/20	
5	66.0	28.8	16/20	
6	56.8	38.4	11/20	
7	10.2	33	8/20	
12	7.4	19.6	8/20	
C2 (Vybromycine treatment; 40 mice)				Group C1 (20 mice)% (survival) 20
-1	7.0	20.4	20/20	m.t.d. = 6.7
0	7.0	18.8	20/20	
1	11.6	12.6	20/20	
3	60.0	10.8	20/20	
5	62.0	16.0	19/20	
6	84.4	34.0	15/20	
7	64.0	30.6	5/20	
8	30.0	26.0	4/20	
12	17.8	22.2	4/20	
D2 (Terramycine treatment; 40 mice)				Group D1 (20 mice)% (survival) 15
-1	7.2	18.8	20/20	m.t.d. = 6.53
0	7.0	17.0	20/20	
1	21.8	15.2	20/20	
3	112.0	25.6	20/20	
5	84.0	26.0	19/20	
6	80.0	36.2	11/20	
7	76.0	28.0	6/20	
8	42.0	20.0	3/20	
12	16.0	18.0	3/20	

[0520] The results set forth in Table 13 show that in the virus control group A2, the concetration of IL-1 increased 24-fold during the course of the disease (from the day 0 until the day 7), and the concentration of TNF increased 7-fold; m.t.d. in this group was 5.5 days and all animals died. In group B2, which was treated with tetracycline therapy, 40% of the animals survived (the m.t.d. of 5.84 is not statistically different from group A2). The concentration of IL-1 increased 10-fold by day 5 of the disease, the concentration of TNF increased 2-fold. the level of the cytokines in the serum of the animals of this group was statistically lower than in the control A2 group. In group C2, which was treated vibromycline, 20% of the animals survived, m.t.d. was 6.7 statistically higher than in the control A2 group. The concentration of IL-1 increased 12-fold by day 6 of the infection, and the concentration of TNF increased 3-fold. The level of cytokines in the serum of the animals of this group was statistically lower than in the control A2 group. In group D2, which was treated with terramycine, 15% of the animals survived, m.t.d. was 6.53, which is statistically longer in the control A2 group. The concentration of IL-1 increased 16-fold by day 3 of the disease and stayed at this level until the day 7. The concentration of TNF increased 2-fold by day 6 of the disease. The levels of the cytokines in the serum of the animals in this group were statistically lower than in the control group A2. Soluble tetracycline was most effective.

EXAMPLE 5

Treatment of the Dengue Virus Infection with Various Tetracyclines and serum

[0521] Virus

[0522] Dengue virus, type 2. All work with infectious virus was performed in the maximum-containment biosafety level-3 (BSL-3) of the SRC VB))Vector)). This virus was amplified in the brain of the suckling mice and was collected to produce stocks. This stock virus suspension was stored at -40 C., contained 6.8 LD_{50}/ml (in the mice BALB/c by intraperitoneal challenge). For infecting mice we used 5 LD_{50} virus.

[0523] Animals

[0524] 4-week-old BALB/c mice (haplotype H-2d), which weighed 12-14 grams, were used in the experiments with Dengue virus. The animals were received from the vivarium of SRC VB ((Vector)) and kept on a standard ration.

[0525] RT-PCR Procedure

[0526] Primers for Dengue virus type 2 detection were: Upper 5'AATATGCTGAAACGCGAGAGAAACCG (position 136-161) SEQ Lower 5'AAGGAACGCCACCAAGGCCATG (position 237-258) SEQ ID No. 24.

[0527] RNA was extracted from the serum of the infected animals (mice) by means of RNeasy Kits (Quiagen, Germany). For RT-PCR Titan-Kits (Berhringer, Germany) were used. Reverse transcription was conducted at 42° C. for 60 min, followed by 40 amplification cycles at 94° C. for 30 sec, at 55° C. for 1 min, and at 68° C. for 2 min, with a final extension at 68° C. for 7 min. Amplification was conducted in 0.2-ml tubes with a model BIS-105M thermocycler (Russia). The virus detection was provided by PCR on the second day after animals infection.

[0528] Experimental Scheme

[0529] Mice of all groups were infected by 5 LD_{50} of Dengue virus.

[0530] Groups A—control groups (only virus).

[0531] Group A1—20 mice—control for mortality.

[0532] Group A2—30 mice—was used for obtaining blood samples on day (0) and on days 1, 3, 5 and 6 post infection. The blood samples were obtained from the orbital sinuses (at every time point 3 mice were used for harvesting blood). All blood samples (500 μl each) were frozen at -70° C. After completion of the experiment, the concentrations of TNF-α and IL-1β were measured. Groups C, 36 mice, were the Human serum treatment group. Treatment was carried out with the Human serum stimulated by Vibromycine. The Human serum was obtained from the blood of a human administered vibromycine (150 mg) orally twice a day (every 12 hours). The human blood was taken on the second and the third day after the beginning of the stimulation. The concentration in the human serum of IL-1RA was 184 pg/ml, and the concentration of sTNFrI was 950 pg/ml.

[0533] Treatment of the mice commenced on the third day after viral infecting of the mice and continued until day 8. It

was administered intraperitonealy twice a day in the volume of 200 μl per dose. The dose of the infusing human serum is about 16% of the blood volume of a mouse.

[0534] Groups B—Tetracycline treatment groups.

[0535] Treatment with Tetracycline hydrochloride (100 μg in a volume of 30 μl) was carried out from the third day after virus infection until day 8, twice per day, orally. Tetracycline is more soluble than vibromycine so that is could be administered more readily in solution to the mice.

[0536] Group B1—control for mortality (20 mice).

[0537] Group B2—30 mice—was used for obtaining blood samples on day (0) and days 1, 3, 5, 6 and 12 post infection. Blood samples were obtained from the orbital sinuses (at every time point 3 mice were used for harvesting blood). All blood samples (500 μl each) were frozen −70° C. After completion of the experiment, the concentrations of TNF-α, and IL-1β were measured.

[0538] Groups C

[0539] Group C1—control for mortality. 10 mice.

[0540] Group C2—26 mice—was used for obtaining blood samples on day (0) and days 1, 3, 5 and 12 post infection. Blood samples were obtained from the orbital sinuses (at every time point 3 mice were used for harvesting blood). All blood samples (500 μl) were frozen and −70° C. After completion of the experiment, the concentrations of TNF-α and IL-1β were measured.

[0541] Groups D Control for human serum treatment groups.

[0542] The control for treatment was human serum obtained from the human before the Vibromycine stimulation. This "normal" human serum contained 24.4 pg/ml of IL-1RA and 25.0 pg/ml of sTNFR1. The volume dose and method of infusion were the same as during the Human serum treatment course. Treatment with the normal human serum commenced on the third day after virus infection until day 7, twice per day, intraperitonealy in a volume of 200 μl per dose. The dose of the infusing normal human serum was about 16% of the blood volume of a mouse.

[0543] Group D1—10 mice—control for mortality.

[0544] Group D2—26 mice—was used for obtaining blood samples on day (0) and days 1, 3, 5 and 6 post infection. Blood samples were obtained from the orbital sinuses (at every time point 3 mice were used for harvesting blood). All blood samples (500 μl) were frozen at −70° C. After completion of the experiment, the concentrations of TNF-α and IL-1β were measured.

[0545] Groups E. Treatment with anti-TNFα serum.

[0546] Group E1—10 mice.

[0547] For treatment rabbit serum prepared against the human TNF-α was used. The neutralizing activity of this rabbit's serum was 1 ng/ml. Treatment with anti-TNF-α serum commenced on the third day after virus infection until day 7, twice per day, intraperitonealy in a volume of 200 μl per dose. The dose of the infusing anti-TNF-α serum represented 16% of the blood volume of a mouse.

[0548] Group E2-10 mice.

[0549] The treatment with the normal rabbit serum was carried out from the third day after virus infection until day 6, twice per day, intraperitonealy in a volume of 200 μl per dose. The dose of the infusing normal rabbit serum represented 16% of the blood volume of a mouse.

[0550] Results

[0551] The results of the experiments show that the oral administration of Tetracycline (groups B) for the treatment of the experimental Dengue fever in mice (20 mg/kg, daily) prolongs (statistically significant) the lifetime of the animals, and increases (statistically significant) the number of the surviving mice (Table 14). The data (see Table below) shows that treatment considerably reduces inflammatory cytokines such as TNFα and IL-1β (Table 15). Treatment with stimulated human serum (groups C) containing the increased concentrations of the receptors of the cytokines also prolonged the lifetime of the mice, and increased the number of surviving animals. The results of the treatment by the normal human serum (groups D) did not reveal any significant differences from the results in the Control group A. Hence this data demonstrates the essential role of TNFα in the development of the experimental Dengue fever.

[0552] These results are further confirmed by the results of the anti-TNFα serum treatment (group E1). In this group 60% of all animals survived and the lifetime was significantly higher.

TABLE 14

The average lifetime and the data of the mortality among the treated mice with the experimental Dengue fever

Group	Scheme of Treatment	Survived/died	m.t.d.
A1	virus control	0/20	6.94 + 0.02
B1	Tetracycline treatment	9/11	8.40 ± 0.73*
C1	Human serum (with sTNF RI and IL-1RA) treatment	3/7	8.54 ± 0.42*
D1	<Normal>human serum	0/10	7.00 ± 0.31
E1	Anti-TNF-α serum treatment	4/6	8.70 ± 0.48*,**
E2	Normal rabbit serum treatment	0/10	6.94 ± 0.02

*the difference with the group A is statistically significant (P < 0.1)
**the difference with the group E2 is statistically significant (P < 0.1)

[0553]

TABLE 15

Dynamics of the changes of the concentrations of TNF-α and IL-β in the serum of the animals with the experimental Dengue fever

Group	Scheme of Treatment	Day	IL-1 pg/ml	TNF pg/ml
A2	virus control	0	6.2	8.0
		1	12.1	14.4
		3	32.8	36.8
		5	62.6	116.4
		6	88.4	459.2
B2	Tetracycline treatment	0	6.0	7.8
		1	12.0	13.8
		3	36.0	38.2
		5	48.6	56.2
		6	62.4	156.8
		12	15.6	18.0
		21	5.8	7.4

TABLE 15-continued

Dynamics of the changes of the concentrations of TNF-α and IL-β in the serum of the animals with the experimental Dengue fever

Group	Scheme of Treatment	Day	IL-1 pg/ml	TNF pg/ml
C2	Human serum (with STNFrI IL-1RA) treatment	0	6.2	7.8
		1	12.2	14.0
		3	36.8	35.8
		5	52.4	78.2
		12	18.2	19.2
		21	6.6	7.6
D2	<Normal> human serum treatment	0	7.0	7.6
		1	12.2	13.6
		3	36.4	36.8
		5	60.8	98.2
		6	84.2	320.0

EXAMPLE 6

Treatment of Marburg Virus Infection

[0554] Virus

[0555] Marburg virus strain Popp was received from the Belarussian Institute of Epidemiology and Microbiology. This virus was amplified in Vero E6 cells and the supernatant was collected to produce stocks. This stock virus suspension has been stored at −70° C., contained $10^7$7 PFU/ml. All work with infectious virus was performed in the maximum-containment biosafety level—4 (BSL-4) of the SRC VB (Vector).

[0556] Animals

[0557] Outbred guinea pigs (male) 200-220 grams were used in the experiments with Marburg virus.

[0558] Experimental Scheme

[0559] All animals were divided into groups, each contained 6 animals.

[0560] The guinea pigs were infected by the 5 LD_{50} of the Marburg virus. Animals of the group A were used only for the virus control. Animals of the group B after infection were treated by the human serum (SERUM1) with IgG against Marburg (titer IgG in ELISA 1:80), without IgG against Ebola and sTNFrI (950 pg/ml), TNFα (7.8 pg/ml), IL-1RA (136 pg/ml), IL-1β, ((3 pg/ml), Animals of the group B were given SERUM1 intracardially from day 3 after virus infection until day 14, every day at the following doses:

[0561] 3 day—200 μl

[0562] 4 day—200 μl

[0563] 5 day—400 μl

[0564] 6 day—400 μl

[0565] 7 day—600 μl

[0566] 8 day—600 μl

[0567] 9 day—600 μl

[0568] 10 day—800 μl

[0569] 11 day—800 μl

[0570] 12 day—800 μl

[0571] 13 day—800 μl

[0572] 14 day—800 μl

[0573] Animals of the group C were treated by the human serum with IgG against Marburg virus (titer IgG in ELISA 1:80), without IgG against Ebola, the concentration of TNFα-7.8 pg/ml, sTNFrI-21 pg/ml, IL-1β-3 pg/ml, IL-IRA-24.4 pg/ml Serum 2.

[0574] Animals of the group C were given Serum 2 intracardially from day 3 after virus infecting until day 12, every day, at the following doses:

[0575] 3 day—200 μl

[0576] 4 day—200 μl

[0577] 5 day—400 μl

[0578] 6 day—400 μl

[0579] 7 day—600 μl

[0580] 8 day—600 μl

[0581] 9 day—600 μl

[0582] 10 day—800 μl

[0583] 11 day—800 μl

[0584] 12 day—800 μl

[0585] Animals of the group D were treated with the human serum without antibodies against Marburg virus and without antibodies against Ebola virus, and with sTNFrI-880 pg/ml,TNFα-7.2 pg/ml, IL-1β-3 pg/ml, IL-1RA-146 pg/ml (Serum 3).

[0586] Animals of group D were given Serum 3 intracardially from 3 day after virus infecting until 12 day, every day, at the following doses:

[0587] 3 day—200 μl

[0588] 4 day—200 μl

[0589] 5 day—400 μl

[0590] 6 day—40 μl

[0591] 7 day—600 μl

[0592] 8 day—600 μl

[0593] 9 day—600 μl

[0594] 10 day—800 μl

[0595] 11 day—800 μl

[0596] 12 day—800 μl

[0597] Animals of the group E were treated with human serum without the antibodies against Marburg and Ebola viruses, and the concentrations of TNF α-7.0 pg/ml, sTNFrI-20pg/ml, IL-1β-3 pg/ml, IL-1RA-20 pg/ml (SERUM 4). Animals of the group E were given Serum 4 intracardially from 3 days after virus, injecting every day, until 12 day, at the following doses:

[0598] 3 day—200 μl

[0599] 4 day—200 μl

[0600] 5 day—400 μl

[0601] 6 day—400 μl

[0602] 7 day—600 μl

[0603] 8 day—600 μl

[0604] 9 day—600 μl

[0605] 10 day—800 μl

[0606] 11 day—800 μl

[0607] 12 day—800 μl

[0608] On the third day after the challenge by the Marburg virus the blood samples taken from all infected guinea pigs (groups A, B, C, D, E) were tested by RT-PCR. This RT-PCR test was performed for the confirmation of the virus infection and showed positive amplification using a cDNA segment of Marburg virus with the approximate size about 420 bp. Detection of the virus by the PCR method in the blood samples performed before the challenge (0 day) showed no Marburg virus.

[0609] On day 7 a positive result by RT-PCR test was obtained. On the 27th day after the challenge, no Marburg virus was detected in the blood samples of the surviving animals.

TABLE 16

Mortality, average lifetime among the infected by the Marburg virus guinea pigs

Group	Serum treatment	Survived/total amount	% of survival	M.T.D.
A	control: only virus	0/6	0%	11.49 + 0.64
B	Serum 1	4/6	66%	13.51 + 0.80*
C	Serum 2	0/6	0%	11.90 + 0.48
D	Serum 3	1/6	16%	11.73 + 0.53
E	Serum 4	0/6	0%	11.62 + 0.48

*statistically significant (P(0.01)

[0610] Results

[0611] All guinea pigs in groups A, C and E died, and the average lifetime was not statistically different from the control group A. In the animals of the group B treated by with SERUM1, which contains antibodies against Marburg virus and soluble receptors sTNFR and IL-IRA, a tendency of increasing survival of animals was observed and the prolongation of lifetime was statistically significant. Human soluble receptors (sTNFR1 and IL-IRA) were detected in the blood samples of the treated guinea pigs on day (0) before infecting (as a control) and on day 7 after infecting with the Marburg virus, and on the 27th day among the survived guinea pigs. The detection was performed using ELISA-kits of R&D Production. The human soluble receptors sTNFR1 and IL-IRA were detected in the blood of the animals. Without being bound by any theory, it appears that these receptors were used for the neutralization of the inflammatory cytokines produced during the development of the Marburg fever in the animals. The serum of the surviving guinea pigs after Marburg infection was used for the detection of the specific IgG by ELISA and Western blot (groups of guinea pigs A, B, C) on days (0), 27 and 35. On day ((0)) no specific IgG was detected. But on day 27 and 35 the specific antibodies against Marburg virus were found at a titer of 1:80. At the same time no antibodies against Ebola virus were detected.

[0612] It appears from the combination of the low titer of the antibodies against the Marburg virus with sufficient concentrations of the soluble receptors of the inflammatory cytokines can influence the development and outcome of the experimental Marburg fever.

EXAMPLE 7

Treatment of *E. Coli* Infection

[0613] Bacterial Strain

[0614] Enterohemorrhagic *Escherichia coli* (EHEC), 0 157:H7 strain, serotype 105282 was used these experiments. The organisms were incubated in LB medium for 24 h ar 37 C. After one passage viable counts were determined by plating on the agar media. Titer of *E. coli* was 10^8 PFU. *E. coli* suspension was prepared by washing the bacterial pellet twice in the phosphate-buffered saline (PBS; pH 7.4).

[0615] Dosage and Method of Infecting

[0616] The bacterial suspension (10^7 PFU) in the volume of 30 μl was infused to the mice intragastrically through the soft polyethylene catheter.

[0617] Mice. 4-week-old male BALB/c mice (halpotype H-2d) were used in the experiments. The blood volume per mouse was approximately 1.2 ml. All animals were divided into the following groups.

[0618] Groups A. Control groups. All animals were infected by *E. coli* suspension.

[0619] Group A-1, 10 mice, control for mortality. Group A2, 20 mice, was used to obtain blood samples on day "0" and day 1, 2, 3, 5 post-infection. Blood samples were obtained from the orbital sinuses (on every time point 3 mice were used for harvesting blood). All blood samples (500 μl each) were frozen −70 C. After the whole experiment had finished, the concentrations of TNF, IL-1 were measured.

[0620] Groups B. Treatment groups (B1 and B2).

[0621] Treatment was carried out with the Human serum containing IL-RA and sTNFrI. The Human serum was obtained from the blood of human taking orally Vibromycine in dose of 150 mg twice per day (every 12 hours). The Human blood was taken on the second day and the third day after the beginning of taking antibiotic. The concentration in the Human serum of IL-1RA was 184 pg/ml, and the concentration of sTNFrI was 950 pg/ml. The treatment was started from the second day after bacterial infecting of the mice and continued until 9 day, twice per day, intraperitoneally, in the volume of 200 μl per dose. The dose of the transfusing Human serum presented 16% of the blood volume of a mouse.

[0622] Group B1, 10 mice, control for mortality.

[0623] Group B2, 26 mice, was used from obtaining blood samples on day "0" and day 1, 2, 3, 5, 12, 21 post infection. Blood samples were obtained from the orbital sinuses (on every time point 3 mice were used for harvesting blood). All

blood samples (500 μl each) were frozen −70° C. After the whole experiment had finished, the concentrations of TNF, IL-1 were measured.

[0624] Groups C. Control for Treatment groups.

[0625] Treatment was carried out with the ((Normal)) Human serum. The concentration in the ((Normal)) Human serum of IL-1RA was 24.4 pg/ml, and the concentration of sTNFrI was 22 pg/ml. The concentration of IL-1β pg/ml, the concentration of TNFα-7.6 pg/ml. The treatment was started from the second day after bacterial infecting of the mice and continued until 7 day, twice per day, intraperitonealy, in the volume of 200 μl per dose. The does of the transfusing Normal Human serum presented 16% of the blood volume of a mouse. All animals died on day 7 after bacterial infection.

[0626] Group C1, 10 mice, control mortality.

[0627] Group C2, 26 mice, was used to obtain blood samples on day "0" and day 1, 2, 3, 5, 6 post infection. Samples were obtaining from the orbital sinuses (on every time point 3 mice were used for harvesting blood). All blood samples (500 μl each) were frozen −70 C. After the whole experiment had finished, the concentrations of TFNa, IL-1β were measured.

[0628] Results

[0629] The results of the experiments show that infecting the mice with a pathogenic strain of *E. coli* leads to the death of all mice. The clinical manifestations of the experimental disease caused by this strain of *E. coli* have many common features with the experimental fevers in animals such as Dengue, Lassa, and Machupo. The presence of sepsis in the infected animals was confirmed by demonstrating *E. coli* in the blood of the animals on the 6th day after infecting while it was not present before infecting. All infected mice showed intensified production of TNFα and IL-1β. Infusion of normal nonstimulated human serum had no effect on the levels of inflammatory cytokines nor did it prolong the lifetime of the animals or the number of survivors. Treatment with vibromycine stimulated human serum that contained resulting higher concentrations of sTNFrI and IL-1RA provides a statistically significant prolongation of lifetime of the infected mice, the survival of 4 of 10 mice and a decrease in production of the cytokines as sTNFrI and IL-1RA.

TABLE 17

The effects of the treatment of the experimental bacterial shock

Group	Scheme of treatment	Survived/died	m.t.d.
A	*E. coli* control	0/10	5.84 ± 0.19
B	Human serum with sTNF and Il-1RA, stimulated.	4/6	7.14 ± 0.49*
C	Human serum (normal)	0/10	6.36 ± 0.29

*the difference from group A is statistically significant (P(0.05)

[0630]

TABLE 18

Dynamics of the changes of concentrations of TNF-α and IL-1β in the serum of animals with experimental bacterial shock.

Group	Days	IL-1 pg/ml	TNF pg/ml
A2	0	7.8	5.4
	1	15.0	8.0
	2	23.0	10.0
	3	40.0	16.0
	5	190.0	362.0
B2	0	7.2	5.6
	1	17.0	9.0
	2	24.0	11.0
	3	33.0	14.0
	5	86.0	136.0
	12	11.0	10.6
	21	6.2	5.0
C2	0	7.2	5.4
	1	15.0	8.0
	2	24.0	11.0
	3	40.0	17.0
	5	172.0	316.0
	6	236.0	488.0

EXAMPLE 8

In Nitro Activation of Mononuclear Human Cells by Antibiotics

[0631] 1. Cells

[0632] Mononuclear cells were obtained from human blood, which had been collected in tubes with Heparin (5 ED heparin/ml) and centrifuged on Hustopaqe (p=1.077), 1000× g, 30 minutes. Mononuclear cells were washed twice with RPMI-1640 medium (pH 7.2). The concentration of the cells was 5×10^6/ml.

[0633] 2. Activation of Cells

[0634] One portion of the cells was used as control, without any activation (in a volume 2 ml). A second portion was used for the tetracycline activation at a concentration of 0.06 mg/ml (in a volume of 2 ml). The third portion was used for the terramycine activation at a concentration of 0.06 mg/ml (in a volume of 2 ml). The activation continued for 2 hours, and the cells then were washed twice with the medium RPMI-1640 (pH 7.2). A monolayer was formed (2×10^6/ml) and the cells were cultured at 37° C., 95% humidity, atmosphere of 5% of CO_2. Samples of activated mononuclear cells were taken on the third, 6th and 24th hours after the beginning of the contact. The concentrations of sTNFrI and IL-1RA were measured using standard ELISA-kits by R&D Systems.

[0635] The results of the experiment showed that the production of the receptors such as sTNFrI and IL-1RA are induced in vitro using Tetracycline and Terramycine. The production of the receptors by the activated cells was statistically significantly higher than the production by the non-stimulated cells. The concentrations of the receptors obtained in vitro are comparable to the concentrations obtained in vivo and even higher. For example, after vibromycine stimulation, the concentration of receptors in the donor serum (2 persons, on the 24th hour) were IL-1RA

126.8±6.8 pg/ml, sTNFrl 970±28.6 pg/ml (before the stimulation: IL-1RA 20±2.2 pg/ml and sTNFrl 22±3.4 pg/ml). After the tetracycline stimulation the concentrations of the same receptors in the donor serum (2person, at the 24th hour) was 130±6.8 pg/ml and 580±18.2 pg/ml.

TABLE 20

Dynamics of the concentrations of IL-1RA and sTNFrl

Cells	Hours	IL-1RA pg/ml	sTNFrl pg/ml
only Cells	0	27 + 1.4	18 + 1.6
	3	40 + 3.2	68 + 4.8
	6	58 + 4.6	44 +-3.2
	24	44 + 3.4	22 + 2.1
Cells + Terramycine	0	28 + 1.6	18 + 1.4
	3	93 + 6.2	313 + 10.4
	6	220 + 9.4	224 + 9.2
	24	185 + 8.6	264 + 9.6
Cells + Tetracycline	0	22 + 1.4	19 + 1.2
	3	86 + 4.6	185 + 8.4
	6	186 + 8.2	204 + 9.2
	24	140 + 7.6	201 + 8.6

EXAMPLE 9

Treatment of Septic Shock with Plasma from Tetracycline-injected Mice

[0636] 1. Preparation of Plasma from Tetracycline-injected Mice

[0637] Sixty 7-8 week old Balb/c mice (H^{2-4} haplotype) were injected intramuscularly with tetracycline (58 mg/kilo in 0.1 ml of sterile PBS). Plasma (citrated)was collected from these mice at 24 hour postinjection. One 0.2 ml sample of the plasma from these mouse was tested for the presence of IL-1R and TNFα-RI&II. the reminder of the plasma from each mouse was pooled. After removing a small sample from this pool for testing for the above mentioned cytokines, the reminder of the plasma pool was stored at −85° C. until needed.

[0638] Thirty 7-8 week old female Balb/c mice (H^{2-d}) were injected with 0.1 ml of sterile PBS and their plasma was drawn at 24 hour postinjection. A sample of plasma from each mouse was tested for IL-1R and TNFα-RI&II. The remainder of the plasma from this group of mice was pooled. A sample of the pooled plasma was tested for the cytokines as described above.

[0639] 2. Treatment of the Mice with Septic Shock

[0640] Fifty 6-8 week old female Balb/c mice (Haplotype as above) received concurrent intraperitoneal injections of 25 μg of Staphylococcus enterotoxin B (SEB) and 20 mg of galactosamine for the induction of Septic Shock. The mice were divided into the following treatment groups:

[0641] 1) ten mice remained untreated and served as negative controls;

[0642] 2) ten mice received an intramuscular injection of tetracycline (58 mg/kilo) on the day of induction, and on days 1, 2, 3 and 4 postinduction. These mice also received twice daily injections of 0.3 ml of plasma from mice treated with tetracycline on the day of induction and on days 1, 2, 3 and 4 postinduction;

[0643] 3) ten mice received 0.3 ml of plasma from tetracycline-injected mice twice daily on the day of induction and on days 1, 2, 3 and 4 postinduction;

[0644] 4) ten mice received intramuscular injection of tetracycline and 0.3 ml of plasma from tetracycline-injected mice once daily on the day of induction and on days 1, 2, 3 and 4 postinduction; and

[0645] 5) ten mice received 0.3 ml of plasma from PBS-injected mice twice daily on the day of induction and on days 1, 2, 3 and 4 postinduction.

[0646] Ten mice were not induced for septic shock and served as normal controls.

[0647] Mortality among all groups of animals was recorded four times daily for 4 days (96 hours) postinduction.

[0648] All mice without the induced septic shock survived 96 hours postinduction. None of the mice with the septic shock treated with control plasma, i.e., plasma prepared from PBS-infected mice, survived 36 hours postinduction. About 20% of the mice with septic shock that were treated with either tetracycline or tetracycline-stimulated plasma alone survived 96 hours postinduction. About 40% of the mice with septic shock that were treated with tetracycline and tetracycline-stimulated plasma survived 96 hours postinduction. Therefore, combination therapy of tetracycline and tetracycline-stimulated plasma boosts the survival rate of the mice with the SEB-induced septic shock.

EXAMPLE 10

Effects of Plasma from Tetracycline-injected on the Outcome of Septic Shock in Mice and Protocols for Testing of Treatment Hemorrhagic Fevers in a Rodent Model

[0649] Individuals infected with gram negative bacteria such as *Escherichia coli* and *Salmonella typhi* develop a characteristic syndrome that includes acidosis, fever, hypotension, lactate release into the tissues, disseminated intravascular coagulation (DIC) and renal, hepatic and lung injury. These infections and the resulting syndrome can induce a lethal condition called septic shock (SS). Numerous studies have established that this pathologic picture is attributable almost entirely to secretion of TNFα by endotoxin-stimulated macrophages.

[0650] Mouse DIC and SS Models

[0651] Balb/c mice sensitized by administration of D-galactosamine and injected intraperitoneally with Staphylococcus enterotoxin B (SEB) are a well-established model for human septic shock with accompanying disseminated intravascular coagulation. This process is driven by the release of TNFα and IL-1 by antigen-stimulated macrophages. In this mouse model, death usually occurs within 24 hr or antigen challenge.

[0652] Phase I

[0653] 1. Sixty, 7-8 week old female Balb/c mice (H^{24} haplotype) are injected intramuscularly with tetracycline (58 mg/kilo in 0.1 ml of sterile PBS).

[0654] 2. Plasma (citrated) is collected from these mice at 24 hr postinjection. One 0.2 ml sample of

plasma from each mouse is set aside for testing for the presence of IL-1R and TNFα-RI&II, the reminder of the plasma from each mouse is pooled. After removing a small sample from this pool for testing for the cytokines of interest, such as IL-1 an TNFα, the reminder of the plasma pool is be stored at −85° C. until needed.

[0655] 3. Thirty, 7-8 week old female Balb/c mice (H^{2-d}) are injected with 0.1 ml of sterile PBS and their plasma drawn at 24 hr postinjection. A sample of plasma from each mouse will be tested for IL-1R and TNFα-RI&II and the remainder of the plasma from this group of mice will be pooled. A sample of the pooled plasma will be tested for cytokines as above.

[0656] Phase II

[0657] 1. Fifty, 6-8 week old female Balb/c mice (Haplotype as above) receive concurrent ip injections of 25 μg of SEB and 20 mg of galactosamine.

[0658] 10 mice remain untreated and serve as negative controls

[0659] 10 mice receive an im injection of tetracycline (58 mg/kilo) on the day of induction, and on days 1, 2, 3 and 4 postinduction. These mice also receive twice daily injections of 0.3 ml of plasma from mice treated with tetracycline on the day of induction and on days 1, 2, 3 and 4 postinduction.

[0660] 10 mice receive 0.3 ml of plasma from tetracycline-injected mice twice daily on the day of induction and on days 1, 2, 3 and 4 postinduction.

[0661] 10 mice receive im injection of tetracycline and 0.3 ml of serum from tetracycline injected mice once daily on the day of induction and on days 1, 2, 3 and 4 postinduction.

[0662] 10 mice receive 0.3 ml of serum from PBS-injected mice twice daily on the day of induction and on days 1, 2, 3 and 4 postinduction.

[0663] 2. Ten mice as described above are not treated for induction of Septic Shock and will serve as normal controls.

[0664] 3. Mortality among all groups of animals is recorded four times daily.

[0665] Design of Experiment

[0666] Investigation of Treatment of Yellow Fever Infection

[0667] 1. Virus—Yellow fever—strain "Dakkar", the stock virus suspension after passage sulking mice.

[0668] 2. Animals—BALB/c, male, 4 weeks age, 140 animals.

[0669] Steps

[0670] 1. Preparation of serum from mice after by injections of Doxycycline, (70 mice for group)

[0671] 2. For mice infection used 5 LD_{50} of YFV.

[0672] group A—control for YFV without treatment—10 mice.

[0673] group B—treatment of YFV by Doxycycline from the third day after infection, every day.

[0674] group C—treatment of YFV by Doxycycline from the third day after infection every 12 h.

[0675] group D—treatment of YFV by serum (with IL-1RA and sTNF) from the third day after infection, every day.

[0676] group E—treatment of YFV by serum (with IL-1RA and sTNF) from the third day after infection every 12 h.

[0677] group F—control virus: for detection soluble receptors (sTNF, IL-1RA) and cytokines (TNF and IL-1) in blood after infection (days 1, 2, 3, 4, 5, 6)—20 mice.

[0678] Investigation of Treatment of Lassa Fever Infection

[0679] 1. Virus—Lassa fever—strain "Josiah", the stock virus suspension after passage suckling mice.

[0680] 2. Animals—CBA/calac, male, 4 weeks age, 140 animals.

[0681] Steps

[0682] 1. Preparation of serum from mice after by injections of Doxycycline. (80 mice for group)

[0683] 2. For mice infection used 10 LD_{50} of LFV.

[0684] group A—control for LFV without treatment—20 mice.

[0685] group B—treatment of LFV by serum (with IL-1RA and sTNF) from the third day after infection, every day (20 mice).

[0686] group D—treatment of LFV by serum (with IL-1RA and sTNF) from the third day after infection every 12 h (20 mice).

EXAMPLE 11

Assays for TNF and IL-1 Receptors

[0687] Assays for IL-1 Receptors

[0688] Numerous bioassays used to detect and quantitate IL-1Ra are known. An assay used herein to determine IL-1Ra in blood and blood-derived fractions that have been treated with tetracycline or tetracycline-like compounds is the Quantikine IL-1ra mmunoassay, which is solid phase ELISA designed to measure IL-1Ra in cell culture supernate, serum, and plasma. It contains *E. coli*-derived recombinant human IL-1Ra as well as antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate the recombinant human IL-1ra. Results obtained during natural human IL-1ra showed linear curves that were parallel to the standard curves obtained using the *E.coli*-expressed Quantikine kit standards. These results indicate that the Quantikine Immunoassay kit can be used to determine relative mass values for natural human IL-1ra.

[0689] Principle of the Assay

[0690] This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-1Ra has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IL-1Ra present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-1Ra is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IL-1Ra bound in the initial step. The color development is stopped and the intensity of the color is measured.

[0691] Assays for TNFs

[0692] Bioassays for sTNFR If typically involves measurement of the inhibitory effect of the soluble receptor on the cytotoxic activity TNF-α on a susceptible cell line. The Quantikine human sTNF RI Immunoassay is a solid phase ELISA designed to measure sTNF RI in cell culture supernate, serum, plasma and urine. It contains *E. coli*-expressed, recombinant human sTNF RI, as well as antibodies raised against this polypeptide. The recombinant protein represents the non-glycosylated, N-terminal methionyl form of the naturally occurring human soluble Type I receptor for TNF with an apparent molecular weight of approximately 18.6 kDa. This immunoassay has been shown to accurately quantitate the recombinant sTNF RI. Results obtained on samples containing natural sTNF RI showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that Quantikine Immunoassay kit can be used to determine relative mass values of natural sTNF RI. Since the measurement of human sTNF RI by this immunoassay is relatively insensitive to added TNF-α or TNF-62 , it is probable that this measurement corresponds to the total amount of the soluble receptor present in samples, i.e., the total amount of free receptor plus the total amount of receptor bound to TNF.

[0693] Principle of the Assay

[0694] This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for sTNF RI has been pre-coated onto a microplate. Standards and samples are pipetted into the wells an any sTNF RI present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for sTNF RI is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of sTNF RI bound in the initial step. The color development is stopped and the intensity of the color is measured.

TABLE 21

Exemplary levels of IL-1, TNF, IL-1RA and sTNF RI in samples from normal volunteers

Sample	IL-1	TNF pg/ml	IL-IRA	sTNF RI
1. subject 1 serum 10/22	3 pg/ml	7.8	241.6	
2. subject 1 serum 12/06	<3 pg/mL	7.8	136.0	950
3. subject 1 serum 12/07	<3 pg/mL	7.8	100.8	970
4. subject 1 serum 12/08	<3 pg/mL	7.8	184.8	875
5. subject 1 plasma 12/01	<3 pg/mL	7.8	140.8	575
6. subject 1 plasma 12/03	<3 pg/mL	7.8	82.4	825
7. subject 1 plasma 12/07	<3 pg/mL	7.8	140.8	600
8. subject 2 serum 12/06	3 pg/mL	8.6	140.8	1650
9. subject 2 serum 12/07	3.9 pg/mL	8.6	164.0	1650
10. subject 2 serum 12/08	<3 pg/mL	8.8	160.0	1750
11. Human IgG	3 pg/mL	7.8	24.4	21.0
12. Swiss	3.9 pg/mL	7.8	31.2	31.2
13. Human-Indonesia	3 pg/mL	8.8	568	2200

[0695] Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.

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Asp Ala Lys Ile Thr Val Ile Leu Arg Ile Ser Lys Thr Gln Leu Tyr
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Glu Ile Pro Lys Thr Ile Thr Gly Ser Glu Thr Asn Leu Leu Phe Phe
210 215 220

Trp Glu Thr His Gly Thr Lys Asn Tyr Phe Thr Ser Val Ala His Pro
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Gln Leu Arg Ile Ser Asp His His Tyr Ser Lys Gly Phe Arg Gln Ala
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Ala Ser Val Val Val Ala Met Asp Lys Leu Arg Lys Met Leu Val Pro
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Cys Pro Gln Thr Phe Gln Glu Asn Asp Leu Ser Thr Phe Phe Pro Phe
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Ser Gln Gln Lys Ser Leu Val Met Ser Gly Pro Tyr Glu Leu Lys Ala
    130                 135                 140

Leu His Leu Gln Gly Gln Asp Met Glu Gln Gln Val Val Phe Ser Met
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Ser Phe Val Gln Gly Glu Glu Ser Asn Asp Lys Ile Pro Val Ala Leu
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Gly Leu Lys Glu Lys Asn Leu Tyr Leu Ser Cys Val Leu Lys Asp Asp
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Lys Lys Met Glu Lys Arg Phe Val Phe Asn Lys Ile Glu Ile Asn Asn
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Lys Leu Glu Phe Glu Ser Ala Gln Phe Pro Asn Trp Tyr Ile Ser Thr
225                 230                 235                 240

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Leu Trp Phe Val Pro Ala Lys Val Glu Asp Ser Gly His Tyr Tyr Cys
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Val Val Arg Asn Ser Ser Tyr Cys Leu Arg Ile Lys Ile Ser Ala Lys
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Phe Val Glu Asn Glu Pro Asn Leu Cys Tyr Asn Ala Gln Ala Ile Phe
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His Met Ile Gly Ile Cys Val Thr Leu Thr Val Ile Ile Val Cys Ser
            340                 345                 350

Val Phe Ile Tyr Lys Ile Phe Lys Ile Asp Ile Val Leu Trp Tyr Arg
        355                 360                 365

Asp Ser Cys Tyr Asp Phe Leu Pro Ile Lys Ala Ser Asp Gly Lys Thr
    370                 375                 380

Tyr Asp Ala Tyr Ile Leu Tyr Pro Lys Thr Val Gly Glu Gly Ser Thr
385                 390                 395                 400

Ser Asp Cys Asp Ile Phe Val Phe Lys Val Leu Pro Glu Val Leu Glu
                405                 410                 415

Lys Gln Cys Gly Tyr Lys Leu Phe Ile Tyr Gly Arg Asp Asp Tyr Val
            420                 425                 430

Gly Glu Asp Ile Val Glu Val Ile Asn Glu Asn Val Lys Lys Ser Arg
        435                 440                 445

Arg Leu Ile Ile Ile Leu Val Arg Glu Thr Ser Gly Phe Ser Trp Leu
    450                 455                 460

Gly Gly Ser Ser Glu Glu Gln Ile Ala Met Tyr Asn Ala Leu Val Gln
465                 470                 475                 480

Asp Gly Ile Lys Val Val Leu Leu Glu Leu Glu Lys Ile Gln Asp Tyr
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Glu Lys Met Pro Glu Ser Ile Lys Phe Ile Lys Gln Lys His Gly Ala
            500                 505                 510

Ile Arg Trp Ser Gly Asp Phe Thr Gln Gly Pro Gln Ser Ala Lys Thr
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Arg Phe Trp Lys Asn Val Arg Tyr His Met Pro Val Gln Arg Arg Ser
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<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<223> OTHER INFORMATION: Interleukin-1 receptor, Type II precursor
<300> PUBLICATION INFORMATION:
<308> DATABASE ACCESSION NUMBER: P27930/GenBank
<309> DATABASE ENTRY DATE: 2001-08-20

<400> SEQUENCE: 4

Met Leu Arg Leu Tyr Val Leu Val Met Gly Val Ser Ala Phe Thr Leu
1 5 10 15

Gln Pro Ala Ala His Thr Gly Ala Ala Arg Ser Cys Arg Phe Arg Gly
20 25 30

Arg His Tyr Lys Arg Glu Phe Arg Leu Glu Gly Glu Pro Val Ala Leu
35 40 45

Arg Cys Pro Gln Val Pro Tyr Trp Leu Trp Ala Ser Val Ser Pro Arg
50 55 60

Ile Asn Leu Thr Trp His Lys Asn Asp Ser Ala Arg Thr Val Pro Gly
65 70 75 80

Glu Glu Glu Thr Arg Met Trp Ala Gln Asp Gly Ala Leu Trp Leu Leu
85 90 95

Pro Ala Leu Gln Glu Asp Ser Gly Thr Tyr Val Cys Thr Thr Arg Asn
100 105 110

Ala Ser Tyr Cys Asp Lys Met Ser Ile Glu Leu Arg Val Phe Glu Asn
115 120 125

Thr Asp Ala Phe Leu Pro Phe Ile Ser Tyr Pro Gln Ile Leu Thr Leu
130 135 140

Ser Thr Ser Gly Val Leu Val Cys Pro Asp Leu Ser Glu Phe Thr Arg
145 150 155 160

Asp Lys Thr Asp Val Lys Ile Gln Trp Tyr Lys Asp Ser Leu Leu Leu
165 170 175

Asp Lys Asp Asn Glu Lys Phe Leu Ser Val Arg Gly Thr Thr His Leu
180 185 190

Leu Val His Asp Val Ala Leu Glu Asp Ala Gly Tyr Tyr Arg Cys Val
195 200 205

Leu Thr Phe Ala His Glu Gly Gln Gln Tyr Asn Ile Thr Arg Ser Ile
210 215 220

Glu Leu Arg Ile Lys Lys Lys Lys Glu Glu Thr Ile Pro Val Ile Ile
225 230 235 240

Ser Pro Leu Lys Thr Ile Ser Ala Ser Leu Gly Ser Arg Leu Thr Ile
245 250 255

Pro Cys Lys Val Phe Leu Gly Thr Gly Thr Pro Leu Thr Thr Met Leu
260 265 270

Trp Trp Thr Ala Asn Asp Thr His Ile Glu Ser Ala Tyr Pro Gly Gly
275 280 285

Arg Val Thr Glu Gly Pro Arg Gln Glu Tyr Ser Glu Asn Asn Glu Asn
290 295 300

Tyr Ile Glu Val Pro Leu Ile Phe Asp Pro Val Thr Arg Glu Asp Leu
305 310 315 320

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His Met Asp Phe Lys Cys Val Val His Asn Thr Leu Ser Phe Gln Thr
325 330 335

Leu Arg Thr Thr Val Lys Glu Ala Ser Ser Thr Phe Ser Trp Gly Ile
340 345 350

Val Leu Ala Pro Leu Ser Leu Ala Phe Leu Val Leu Gly Gly Ile Trp
355 360 365

Met His Arg Arg Cys Lys His Arg Thr Gly Lys Ala Asp Gly Leu Thr
370 375 380

Val Leu Trp Pro His His Gln Asp Phe Gln Ser Tyr Pro Lys
385 390 395

<210> SEQ ID NO 5
<211> LENGTH: 177
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<223> OTHER INFORMATION: Interleukin-1 Receptor Antagonist Protein Precursor (IL-1RA; ICIL-1RA; IRAP)
<300> PUBLICATION INFORMATION:
<308> DATABASE ACCESSION NUMBER: P18510/GenBank
<309> DATABASE ENTRY DATE: 2001-08-20

<400> SEQUENCE: 5

Met Glu Ile Cys Arg Gly Leu Arg Ser His Leu Ile Thr Leu Leu Leu
1 5 10 15

Phe Leu Phe His Ser Glu Thr Ile Cys Arg Pro Ser Gly Arg Lys Ser
20 25 30

Ser Lys Met Gln Ala Phe Arg Ile Trp Asp Val Asn Gln Lys Thr Phe
35 40 45

Tyr Leu Arg Asn Asn Gln Leu Val Ala Gly Tyr Leu Gln Gly Pro Asn
50 55 60

Val Asn Leu Glu Glu Lys Ile Asp Val Val Pro Ile Glu Pro His Ala
65 70 75 80

Leu Phe Leu Gly Ile His Gly Gly Lys Met Cys Leu Ser Cys Val Lys
85 90 95

Ser Gly Asp Glu Thr Arg Leu Gln Leu Glu Ala Val Asn Ile Thr Asp
100 105 110

Leu Ser Glu Asn Arg Lys Gln Asp Lys Arg Phe Ala Phe Ile Arg Ser
115 120 125

Asp Ser Gly Pro Thr Thr Ser Phe Glu Ser Ala Ala Cys Pro Gly Trp
130 135 140

Phe Leu Cys Thr Ala Met Glu Ala Asp Gln Pro Val Ser Leu Thr Asn
145 150 155 160

Met Pro Asp Glu Gly Val Met Val Thr Lys Phe Tyr Phe Gln Glu Asp
165 170 175

Glu

<210> SEQ ID NO 6
<211> LENGTH: 176
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<223> OTHER INFORMATION: IL-1 receptor intracellular ligand protein comprising amino acid sequence
<300> PUBLICATION INFORMATION:
<310> PATENT DOCUMENT NUMBER: 5,817,476
<311> PATENT FILING DATE: 1995-06-07
<312> PUBLICATION DATE: 1998-10-06

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<400> SEQUENCE: 6

```
Ile Pro Arg Val Asp Leu Arg Val Trp Gln Asp Cys Cys Glu Asp Cys
 1               5                   10                  15

Arg Thr Arg Gly Gln Phe Asn Ala Phe Ser Tyr His Phe Arg Gly Arg
            20                  25                  30

Arg Ser Leu Glu Phe Ser Tyr Gln Glu Asp Lys Pro Thr Lys Lys Thr
        35                  40                  45

Arg Pro Arg Lys Ile Pro Ser Val Gly Arg Gln Gly Glu His Leu Ser
    50                  55                  60

Asn Ser Thr Ser Ala Phe Ser Thr Arg Ser Asp Ala Ser Gly Thr Asn
65                  70                  75                  80

Asp Phe Arg Glu Phe Val Leu Glu Met Gln Lys Thr Ile Thr Asp Leu
                85                  90                  95

Arg Thr Gln Ile Lys Lys Leu Glu Ser Arg Leu Ser Thr Thr Glu Cys
            100                 105                 110

Val Asp Ala Gly Gly Glu Ser His Ala Asn Asn Thr Lys Trp Lys Lys
        115                 120                 125

Asp Ala Cys Thr Ile Cys Glu Cys Lys Asp Gly Gln Val Thr Cys Phe
    130                 135                 140

Val Glu Ala Cys Pro Pro Ala Thr Cys Ala Val Pro Val Asn Ile Pro
145                 150                 155                 160

Gly Ala Cys Cys Pro Val Cys Leu Gln Lys Arg Ala Glu Glu Lys Pro
                165                 170                 175
```

<210> SEQ ID NO 7
<211> LENGTH: 320
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<223> OTHER INFORMATION: IL-1 receptor intracellular ligand protein
      comprising amino acid sequence
<300> PUBLICATION INFORMATION:
<310> PATENT DOCUMENT NUMBER: 5,817,476
<311> PATENT FILING DATE: 1995-06-07
<312> PUBLICATION DATE: 1998-10-06

<400> SEQUENCE: 7

```
Lys Lys Gly Gly Lys Thr Glu Gln Asp Gly Tyr Gln Lys Pro Thr Asn
 1               5                   10                  15

Lys His Phe Thr Gln Ser Pro Lys Lys Ser Val Ala Asp Leu Leu Gly
            20                  25                  30

Ser Phe Glu Gly Lys Arg Arg Leu Leu Leu Ile Thr Ala Pro Lys Ala
        35                  40                  45

Glu Asn Asn Met Tyr Val Gln Gln Arg Asp Glu Tyr Leu Glu Ser Phe
    50                  55                  60

Cys Lys Met Ala Thr Arg Lys Ile Ser Val Ile Thr Ile Phe Gly Pro
65                  70                  75                  80

Val Asn Asn Ser Thr Met Lys Ile Asp His Phe Gln Leu Asp Asn Glu
                85                  90                  95

Lys Pro Met Arg Val Val Asp Asp Glu Asp Leu Val Asp Gln Arg Leu
            100                 105                 110

Ile Ser Glu Leu Arg Lys Glu Tyr Gly Met Thr Tyr Asn Asp Phe Phe
        115                 120                 125

Met Val Leu Thr Asp Val Asp Leu Arg Val Lys Gln Tyr Tyr Glu Val
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    130                 135                 140

Pro Ile Thr Met Lys Ser Val Phe Asp Leu Ile Asp Thr Phe Gln Ser
145                 150                 155                 160

Arg Ile Lys Asp Met Glu Lys Gln Lys Lys Glu Gly Ile Val Cys Lys
                165                 170                 175

Glu Glu Val Gly Gly Val Leu Glu Leu Phe Pro Ile Asn Gly Ser Ser
            180                 185                 190

Val Val Glu Arg Glu Asp Val Pro Ala His Leu Val Lys Asp Ile Arg
        195                 200                 205

Asn Tyr Phe Gln Val Ser Pro Glu Tyr Phe Ser Met Leu Leu Val Gly
    210                 215                 220

Lys Asp Gly Asn Val Lys Ser Trp Tyr Pro Ser Pro Met Trp Ser Met
225                 230                 235                 240

Val Ile Val Tyr Asp Leu Ile Asp Ser Met Gln Leu Arg Arg Gln Glu
                245                 250                 255

Met Ala Ile Gln Gln Ser Leu Gly Met Arg Cys Gln Lys Met Ser Met
            260                 265                 270

Gln Ala Met Val Thr Ile Val Thr Thr Lys Asp Thr Arg Met Val Thr
        275                 280                 285

Arg Met Thr Thr Val Ile Met Arg Val Ile Thr Met Asp Thr Leu Thr
    290                 295                 300

Glu Gln Lys Tyr Val Thr Leu Asp Ser Ala Ser Phe Leu Cys Ser Cys
305                 310                 315                 320


<210> SEQ ID NO 8
<211> LENGTH: 251
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<223> OTHER INFORMATION: IL-1 receptor intracellular ligand protein
      comprising amino acid sequence
<300> PUBLICATION INFORMATION:
<310> PATENT DOCUMENT NUMBER: 5,817,476
<311> PATENT FILING DATE: 1995-06-07
<312> PUBLICATION DATE: 1998-10-06

<400> SEQUENCE: 8

Lys Asn Phe Phe Leu Thr Asn Arg Ala Arg Glu Arg Ser Asp Thr Phe
  1               5                  10                  15

Ile Asn Leu Arg Glu Val Leu Asn Arg Phe Lys Leu Pro Pro Gly Glu
             20                  25                  30

Tyr Ile Leu Val Pro Ser Thr Phe Glu Pro Asn Lys Asp Gly Asp Phe
         35                  40                  45

Cys Ile Arg Val Phe Ser Glu Lys Lys Ala Asp Tyr Gln Ala Val Asp
     50                  55                  60

Asp Glu Ile Glu Ala Asn Leu Glu Glu Phe Asp Ile Ser Glu Asp Asp
 65                  70                  75                  80

Ile Asp Asp Gly Phe Arg Arg Leu Phe Ala Gln Leu Ala Gly Glu Asp
                 85                  90                  95

Ala Glu Ile Ser Ala Phe Glu Leu Gln Thr Ile Leu Arg Arg Val Leu
            100                 105                 110

Ala Lys Arg Gln Asp Ile Lys Ser Asp Gly Phe Ser Ile Glu Thr Cys
        115                 120                 125

Lys Ile Met Val Asp Met Leu Asp Ser Asp Gly Ser Gly Lys Leu Gly
    130                 135                 140
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Leu Lys Glu Phe Tyr Ile Leu Trp Thr Lys Ile Gln Lys Tyr Gln Lys
145                 150                 155                 160

Ile Tyr Arg Glu Ile Asp Val Asp Arg Ser Gly Thr Met Asn Ser Tyr
                165                 170                 175

Glu Met Arg Lys Ala Leu Glu Glu Ala Gly Phe Lys Met Pro Cys Gln
            180                 185                 190

Leu His Gln Val Ile Val Ala Arg Phe Ala Asp Asp Gln Leu Ile Ile
        195                 200                 205

Asp Phe Asp Asn Phe Val Arg Cys Leu Val Arg Leu Glu Thr Leu Phe
    210                 215                 220

Lys Ile Phe Lys Gln Leu Asp Pro Glu Asn Thr Gly Thr Ile Glu Leu
225                 230                 235                 240

Asp Leu Ile Ser Trp Leu Cys Phe Ser Val Leu
                245                 250


<210> SEQ ID NO 9
<211> LENGTH: 700
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<223> OTHER INFORMATION: IL-1 receptor intracellular ligand protein
      comprising amino acid sequence
<300> PUBLICATION INFORMATION:
<310> PATENT DOCUMENT NUMBER: 5,817,476
<311> PATENT FILING DATE: 1995-06-07
<312> PUBLICATION DATE: 1998-10-06

<400> SEQUENCE: 9

Met Ala Gly Ile Ala Ala Lys Leu Ala Lys Asp Arg Glu Ala Ala Glu
  1               5                  10                  15

Gly Leu Gly Ser His Glu Arg Ala Ile Lys Tyr Leu Asn Gln Asp Tyr
             20                  25                  30

Glu Ala Leu Arg Asn Glu Cys Leu Glu Ala Gly Thr Leu Phe Gln Asp
         35                  40                  45

Pro Ser Phe Pro Ala Ile Pro Ser Ala Leu Gly Phe Lys Glu Leu Gly
     50                  55                  60

Pro Tyr Ser Ser Lys Thr Arg Gly Met Arg Trp Lys Arg Pro Thr Glu
 65                  70                  75                  80

Ile Cys Ala Asp Pro Gln Phe Ile Ile Gly Gly Ala Thr Arg Thr Asp
                 85                  90                  95

Ile Cys Gln Gly Ala Leu Gly Asp Cys Trp Leu Leu Ala Ala Ile Ala
            100                 105                 110

Ser Leu Thr Leu Asn Glu Glu Ile Leu Ala Arg Val Val Pro Leu Asn
        115                 120                 125

Gln Ser Phe Gln Glu Asn Tyr Ala Gly Ile Phe His Phe Gln Phe Trp
    130                 135                 140

Gln Tyr Gly Glu Trp Val Glu Val Val Val Asp Asp Arg Leu Pro Thr
145                 150                 155                 160

Lys Asp Gly Glu Leu Leu Phe Val His Ser Ala Glu Gly Ser Glu Phe
                165                 170                 175

Trp Ser Ala Leu Leu Glu Lys Ala Tyr Ala Lys Ile Asn Gly Cys Tyr
            180                 185                 190

Glu Ala Leu Ser Gly Gly Ala Thr Thr Glu Gly Phe Glu Asp Phe Thr
        195                 200                 205
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Gly Gly Ile Ala Glu Trp Tyr Glu Leu Lys Lys Pro Pro Pro Asn Leu
    210                 215                 220

Phe Lys Ile Ile Gln Lys Ala Leu Gln Lys Gly Ser Leu Leu Gly Cys
225                 230                 235                 240

Ser Ile Asp Ile Thr Ser Ala Ala Asp Ser Glu Ala Ile Thr Phe Gln
                245                 250                 255

Lys Leu Val Lys Gly His Ala Tyr Ser Val Thr Gly Ala Glu Glu Val
            260                 265                 270

Glu Ser Asn Gly Ser Leu Gln Lys Leu Ile Arg Ile Arg Asn Pro Trp
        275                 280                 285

Gly Glu Val Glu Trp Thr Gly Arg Trp Asn Asp Asn Cys Pro Ser Trp
    290                 295                 300

Asn Thr Ile Asp Pro Glu Glu Arg Glu Arg Leu Thr Arg Arg His Glu
305                 310                 315                 320

Asp Gly Glu Phe Trp Met Ser Phe Ser Asp Phe Leu Arg His Tyr Ser
                325                 330                 335

Arg Leu Glu Ile Cys Asn Leu Thr Pro Asp Thr Leu Thr Ser Asp Thr
            340                 345                 350

Tyr Lys Lys Trp Lys Leu Thr Lys Met Asp Gly Asn Trp Arg Arg Gly
        355                 360                 365

Ser Thr Ala Gly Gly Cys Arg Asn Tyr Pro Asn Thr Phe Trp Met Asn
    370                 375                 380

Pro Gln Tyr Leu Ile Lys Leu Glu Glu Glu Asp Glu Asp Glu Glu Asp
385                 390                 395                 400

Gly Glu Ser Gly Cys Thr Phe Leu Val Gly Leu Ile Gln Lys His Arg
                405                 410                 415

Arg Arg Gln Arg Lys Met Gly Glu Asp Met His Thr Ile Gly Phe Gly
            420                 425                 430

Ile Tyr Glu Val Pro Glu Glu Leu Ser Gly Gln Thr Asn Ile His Leu
        435                 440                 445

Ser Lys Asn Phe Phe Leu Thr Asn Arg Ala Arg Glu Arg Ser Asp Thr
    450                 455                 460

Phe Ile Asn Leu Arg Glu Val Leu Asn Arg Phe Lys Leu Pro Pro Gly
465                 470                 475                 480

Glu Tyr Ile Leu Val Pro Ser Thr Phe Glu Pro Asn Lys Asp Gly Asp
                485                 490                 495

Phe Cys Ile Arg Val Phe Ser Glu Lys Lys Ala Asp Tyr Gln Ala Val
            500                 505                 510

Asp Asp Glu Ile Glu Ala Asn Leu Glu Glu Phe Asp Ile Ser Glu Asp
        515                 520                 525

Asp Ile Asp Asp Gly Val Arg Arg Leu Phe Ala Gln Leu Ala Gly Glu
    530                 535                 540

Asp Ala Glu Ile Ser Ala Phe Glu Leu Gln Thr Ile Leu Arg Arg Val
545                 550                 555                 560

Leu Ala Lys Arg Gln Asp Ile Lys Ser Asp Gly Phe Ser Ile Glu Thr
                565                 570                 575

Cys Lys Ile Met Val Asp Met Leu Asp Ser Asp Gly Ser Gly Lys Leu
            580                 585                 590

Gly Leu Lys Glu Phe Tyr Ile Leu Trp Thr Lys Ile Gln Lys Tyr Gln
        595                 600                 605

Lys Ile Tyr Arg Glu Ile Asp Val Asp Arg Ser Gly Thr Met Asn Ser
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    610                 615                 620

Tyr Glu Met Arg Lys Ala Leu Glu Glu Ala Gly Phe Lys Met Pro Cys
625                 630                 635                 640

Gln Leu His Gln Val Ile Val Ala Arg Phe Ala Asp Asp Gln Leu Ile
                645                 650                 655

Ile Asp Phe Asp Asn Phe Val Arg Cys Leu Val Arg Leu Glu Thr Leu
            660                 665                 670

Phe Lys Ile Phe Lys Gln Leu Asp Pro Glu Asn Thr Gly Thr Ile Glu
        675                 680                 685

Leu Asp Leu Ile Ser Trp Leu Cys Phe Ser Val Leu
    690                 695                 700


<210> SEQ ID NO 10
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA which is antisense to human IL-1 beta
<300> PUBLICATION INFORMATION:
<301> AUTHORS: Fujiwara, Toshiyoshi Grimm, Elizabeth A.
<302> TITLE: Specific Inhibition of Interleukin 1 beta Gene Expression
      by an Antisense Oligonucleotide: Obligatory Role of Interleukin 1
      in the Generation of Lymphokine-activated Killer Cells
<303> JOURNAL: Cancer Res.
<304> VOLUME: 52
<306> PAGES: 4954-4959
<307> DATE: 1992-09-15

<400> SEQUENCE: 10

ctcaggtact tctgccat                                                18


<210> SEQ ID NO 11
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA which is antisense to human IL-1 alpha
<300> PUBLICATION INFORMATION:
<301> AUTHORS: Maier, Jeanette A.
      Voulalas, Pamela
      Roeder, David
      Maclag, Thomas
<302> TITLE: Extension of the Life-Span of Human Endothelial Cells by an
      Interleukin-1 alpha Antisense Oligomer
<303> JOURNAL: Science
<304> VOLUME: 249
<306> PAGES: 1570-1574
<307> DATE: 1990-09-28

<400> SEQUENCE: 11

tggatgggca actgatgtga aata                                         24


<210> SEQ ID NO 12
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      phosphorothioate DNA which is antisense to IL-1 receptor
<300> PUBLICATION INFORMATION:
<301> AUTHORS: Miraglia, Loren
      Geiger, Thomas
      Bennett, C. Frank
      Dean, Nicholas M.
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<302> TITLE: Inhibition of Interleukin-1 Type I Receptor Expression in Human Cell-Lines by an Antisense Phosphorothioate Oligodeoxynucleotide
<303> JOURNAL: Int. J. Immunopharmacol.
<304> VOLUME: 18
<305> ISSUE: 4
<306> PAGES: 227-240
<307> DATE: 1996

<400> SEQUENCE: 12

tgtgtcctgc aatcggtggc 20

<210> SEQ ID NO 13
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic phosphodiester or phosphorothioate DNA which is antisense to human IL-1 receptor
<300> PUBLICATION INFORMATION:
<301> AUTHORS: Burch, Ronald M.
Mahan, Lawrence C.
<302> TITLE: Oligonucleotides Antisense to the Interleukin Receptor I mRNA Block the Effects of Interleukin I in Cultured Murine and Human Fibroblasts and in Mice
<303> JOURNAL: J. Clin. Invest.
<304> VOLUME: 88
<306> PAGES: 1190-1196
<307> DATE: 1991

<400> SEQUENCE: 13

tctgagtaac actttcat 18

<210> SEQ ID NO 14
<211> LENGTH: 233
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<223> OTHER INFORMATION: Tumor Necrosis Factor Precursor (TNF-alpha; Cachectin)
<300> PUBLICATION INFORMATION:
<308> DATABASE ACCESSION NUMBER: P01375/GenBank
<309> DATABASE ENTRY DATE: 2001-08-20

<400> SEQUENCE: 14

```
Met Ser Thr Glu Ser Met Ile Arg Asp Val Glu Leu Ala Glu Glu Ala
  1               5                  10                  15

Leu Pro Lys Lys Thr Gly Gly Pro Gln Gly Ser Arg Arg Cys Leu Phe
             20                  25                  30

Leu Ser Leu Phe Ser Phe Leu Ile Val Ala Gly Ala Thr Thr Leu Phe
         35                  40                  45

Cys Leu Leu His Phe Gly Val Ile Gly Pro Gln Arg Glu Glu Phe Pro
     50                  55                  60

Arg Asp Leu Ser Leu Ile Ser Pro Leu Ala Gln Ala Val Arg Ser Ser
 65                  70                  75                  80

Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val Val Ala Asn Pro
                 85                  90                  95

Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg Ala Asn Ala Leu
            100                 105                 110

Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu Val Val Pro Ser
        115                 120                 125

Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe Lys Gly Gln Gly
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    130                 135                 140

Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile Ser Arg Ile Ala
145                 150                 155                 160

Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala Ile Lys Ser Pro
                165                 170                 175

Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys Pro Trp Tyr Glu
            180                 185                 190

Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg Leu
        195                 200                 205

Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe Ala Glu Ser Gly
    210                 215                 220

Gln Val Tyr Phe Gly Ile Ile Ala Leu
225                 230


<210> SEQ ID NO 15
<211> LENGTH: 205
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<223> OTHER INFORMATION: Tumor Necrosis Factor Beta (Lymphotoxin Alpha)
<300> PUBLICATION INFORMATION:
<308> DATABASE ACCESSION NUMBER: P01374/GenBank
<309> DATABASE ENTRY DATE: 2001-08-20

<400> SEQUENCE: 15

Met Thr Pro Pro Glu Arg Leu Phe Leu Pro Arg Val Cys Gly Thr Thr
  1               5                  10                  15

Leu His Leu Leu Leu Leu Gly Leu Leu Leu Val Leu Leu Pro Gly Ala
            20                  25                  30

Gln Gly Leu Pro Gly Val Gly Leu Thr Pro Ser Ala Ala Gln Thr Ala
        35                  40                  45

Arg Gln His Pro Lys Met His Leu Ala His Ser Thr Leu Lys Pro Ala
    50                  55                  60

Ala His Leu Ile Gly Asp Pro Ser Lys Gln Asn Ser Leu Leu Trp Arg
 65                  70                  75                  80

Ala Asn Thr Asp Arg Ala Phe Leu Gln Asp Gly Phe Ser Leu Ser Asn
                85                  90                  95

Asn Ser Leu Leu Val Pro Thr Ser Gly Ile Tyr Phe Val Tyr Ser Gln
            100                 105                 110

Val Val Phe Ser Gly Lys Ala Tyr Ser Pro Lys Ala Thr Ser Ser Pro
        115                 120                 125

Leu Tyr Leu Ala His Glu Val Gln Leu Phe Ser Ser Gln Tyr Pro Phe
    130                 135                 140

His Val Pro Leu Leu Ser Ser Gln Lys Met Val Tyr Pro Gly Leu Gln
145                 150                 155                 160

Glu Pro Trp Leu His Ser Met Tyr His Gly Ala Ala Phe Gln Leu Thr
                165                 170                 175

Gln Gly Asp Gln Leu Ser Thr His Thr Asp Gly Ile Pro His Leu Val
            180                 185                 190

Leu Ser Pro Ser Thr Val Phe Phe Gly Ala Phe Ala Leu
        195                 200                 205


<210> SEQ ID NO 16
<211> LENGTH: 455
<212> TYPE: PRT
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<213> ORGANISM: Homo sapiens
<220> FEATURE:
<223> OTHER INFORMATION: Tumor Necrosis Factor p55 Receptor
<300> PUBLICATION INFORMATION:
<308> DATABASE ACCESSION NUMBER: AAA36753/GenBank
<309> DATABASE ENTRY DATE: 1995-08-03

<400> SEQUENCE: 16

Met Gly Leu Ser Thr Val Pro Asp Leu Leu Leu Pro Leu Val Leu Leu
1               5                   10                  15

Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu Val Pro
            20                  25                  30

His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro Gln Gly Lys
        35                  40                  45

Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys
    50                  55                  60

Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp
65                  70                  75                  80

Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu
                85                  90                  95

Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val
            100                 105                 110

Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys Arg
        115                 120                 125

Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe
    130                 135                 140

Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu
145                 150                 155                 160

Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu
                165                 170                 175

Asn Glu Cys Val Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr
            180                 185                 190

Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser
        195                 200                 205

Gly Thr Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu
    210                 215                 220

Leu Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys
225                 230                 235                 240

Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys Glu
                245                 250                 255

Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala Pro Asn Pro Ser
            260                 265                 270

Phe Ser Pro Thr Pro Gly Phe Thr Pro Thr Leu Gly Phe Ser Pro Val
        275                 280                 285

Pro Ser Ser Thr Phe Thr Ser Ser Ser Thr Tyr Thr Pro Gly Asp Cys
    290                 295                 300

Pro Asn Phe Ala Ala Pro Arg Arg Glu Val Ala Pro Pro Tyr Gln Gly
305                 310                 315                 320

Ala Asp Pro Ile Leu Ala Thr Ala Leu Ala Ser Asp Pro Ile Pro Asn
                325                 330                 335

Pro Leu Gln Lys Trp Glu Asp Ser Ala His Lys Pro Gln Ser Leu Asp
            340                 345                 350

Thr Asp Asp Pro Ala Thr Leu Tyr Ala Val Val Glu Asn Val Pro Pro
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        355                 360                 365

Leu Arg Trp Lys Glu Phe Val Arg Arg Leu Gly Leu Ser Asp His Glu
    370                 375                 380

Ile Asp Arg Leu Glu Leu Gln Asn Gly Arg Cys Leu Arg Glu Ala Gln
385                 390                 395                 400

Tyr Ser Met Leu Ala Thr Trp Arg Arg Arg Thr Pro Arg Arg Glu Ala
                405                 410                 415

Thr Leu Glu Leu Leu Gly Arg Val Leu Arg Asp Met Asp Leu Leu Gly
            420                 425                 430

Cys Leu Glu Asp Ile Glu Glu Ala Leu Cys Gly Pro Ala Ala Leu Pro
        435                 440                 445

Pro Ala Pro Ser Leu Leu Arg
    450                 455


<210> SEQ ID NO 17
<211> LENGTH: 461
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<223> OTHER INFORMATION: Tumor Necrosis Factor p75 Receptor
<300> PUBLICATION INFORMATION:
<308> DATABASE ACCESSION NUMBER: P20333/GenBank
<309> DATABASE ENTRY DATE: 2001-08-20

<400> SEQUENCE: 17

Met Ala Pro Val Ala Val Trp Ala Ala Leu Ala Val Gly Leu Glu Leu
  1               5                  10                  15

Trp Ala Ala Ala His Ala Leu Pro Ala Gln Val Ala Phe Thr Pro Tyr
            20                  25                  30

Ala Pro Glu Pro Gly Ser Thr Cys Arg Leu Arg Glu Tyr Tyr Asp Gln
        35                  40                  45

Thr Ala Gln Met Cys Cys Ser Lys Cys Ser Pro Gly Gln His Ala Lys
    50                  55                  60

Val Phe Cys Thr Lys Thr Ser Asp Thr Val Cys Asp Ser Cys Glu Asp
 65                  70                  75                  80

Ser Thr Tyr Thr Gln Leu Trp Asn Trp Val Pro Glu Cys Leu Ser Cys
                85                  90                  95

Gly Ser Arg Cys Ser Ser Asp Gln Val Glu Thr Gln Ala Cys Thr Arg
            100                 105                 110

Glu Gln Asn Arg Ile Cys Thr Cys Arg Pro Gly Trp Tyr Cys Ala Leu
        115                 120                 125

Ser Lys Gln Glu Gly Cys Arg Leu Cys Ala Pro Leu Arg Lys Cys Arg
    130                 135                 140

Pro Gly Phe Gly Val Ala Arg Pro Gly Thr Glu Thr Ser Asp Val Val
145                 150                 155                 160

Cys Lys Pro Cys Ala Pro Gly Thr Phe Ser Asn Thr Thr Ser Ser Thr
                165                 170                 175

Asp Ile Cys Arg Pro His Gln Ile Cys Asn Val Val Ala Ile Pro Gly
            180                 185                 190

Asn Ala Ser Arg Asp Ala Val Cys Thr Ser Thr Ser Pro Thr Arg Ser
        195                 200                 205

Met Ala Pro Gly Ala Val His Leu Pro Gln Pro Val Ser Thr Arg Ser
    210                 215                 220

Gln His Thr Gln Pro Thr Pro Glu Pro Ser Thr Ala Pro Ser Thr Ser
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-continued

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225                 230                 235                 240

Phe Leu Leu Pro Met Gly Pro Ser Pro Pro Ala Glu Gly Ser Thr Gly
                245                 250                 255

Asp Phe Ala Leu Pro Val Gly Leu Ile Val Gly Val Thr Ala Leu Gly
            260                 265                 270

Leu Leu Ile Ile Gly Val Val Asn Cys Val Ile Met Thr Gln Val Lys
        275                 280                 285

Lys Lys Pro Leu Cys Leu Gln Arg Glu Ala Lys Val Pro His Leu Pro
    290                 295                 300

Ala Asp Lys Ala Arg Gly Thr Gln Gly Pro Glu Gln Gln His Leu Leu
305                 310                 315                 320

Ile Thr Ala Pro Ser Ser Ser Ser Ser Ser Leu Glu Ser Ser Ala Ser
                325                 330                 335

Ala Leu Asp Arg Arg Ala Pro Thr Arg Asn Gln Pro Gln Ala Pro Gly
            340                 345                 350

Val Glu Ala Ser Gly Ala Gly Glu Ala Arg Ala Ser Thr Gly Ser Ser
        355                 360                 365

Asp Ser Ser Pro Gly Gly His Gly Thr Gln Val Asn Val Thr Cys Ile
    370                 375                 380

Val Asn Val Cys Ser Ser Ser Asp His Ser Ser Gln Cys Ser Ser Gln
385                 390                 395                 400

Ala Ser Ser Thr Met Gly Asp Thr Asp Ser Ser Pro Ser Glu Ser Pro
                405                 410                 415

Lys Asp Glu Gln Val Pro Phe Ser Lys Glu Glu Cys Ala Phe Arg Ser
            420                 425                 430

Gln Leu Glu Thr Pro Glu Thr Leu Leu Gly Ser Thr Glu Glu Lys Pro
        435                 440                 445

Leu Pro Leu Gly Val Pro Asp Ala Gly Met Lys Pro Ser
    450                 455                 460
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<210> SEQ ID NO 18
<211> LENGTH: 410
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<223> OTHER INFORMATION: TNF receptor death domain ligand protein comprising amino acid sequence
<300> PUBLICATION INFORMATION:
<310> PATENT DOCUMENT NUMBER: 5,849,501
<311> PATENT FILING DATE: 1995-06-19
<312> PUBLICATION DATE: 1998-12-15

<400> SEQUENCE: 18

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Ser Asn Ala Gly Asp Gly Pro Gly Gly Glu Gly Ser Val His Leu Ala
  1               5                  10                  15

Ser Ser Arg Gly Thr Leu Ser Asp Ser Glu Ile Glu Thr Asn Ser Ala
             20                  25                  30

Thr Ser Thr Ile Phe Gly Lys Ala His Ser Leu Lys Pro Ser Ile Lys
         35                  40                  45

Glu Lys Leu Ala Gly Ser Pro Ile Arg Thr Ser Glu Asp Val Ser Gln
     50                  55                  60

Arg Val Tyr Leu Tyr Glu Gly Leu Leu Gly Lys Glu Arg Ser Thr Leu
 65                  70                  75                  80

Trp Asp Gln Met Gln Phe Trp Glu Asp Ala Phe Leu Asp Ala Val Met
                 85                  90                  95
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-continued

Leu Glu Arg Glu Gly Met Gly Met Asp Gln Gly Pro Gln Glu Met Ile
100 105 110

Asp Arg Tyr Leu Ser Leu Gly Glu His Asp Arg Lys Arg Leu Glu Asp
115 120 125

Asp Glu Asp Arg Leu Leu Ala Thr Leu Leu His Asn Leu Ile Ser Tyr
130 135 140

Met Leu Leu Met Lys Val Asn Lys Asn Asp Ile Arg Lys Lys Val Arg
145 150 155 160

Arg Leu Met Gly Lys Ser His Ile Gly Leu Val Tyr Ser Gln Gln Ile
165 170 175

Asn Glu Val Leu Asp Gln Leu Ala Asn Leu Asn Gly Arg Asp Leu Ser
180 185 190

Ile Trp Ser Ser Gly Ser Arg His Met Lys Lys Gln Thr Phe Val Val
195 200 205

His Ala Gly Thr Asp Thr Asn Gly Asp Ile Phe Phe Met Glu Val Cys
210 215 220

Asp Asp Cys Val Val Leu Arg Ser Asn Ile Gly Thr Val Tyr Glu Arg
225 230 235 240

Trp Trp Tyr Glu Lys Leu Ile Asn Met Thr Tyr Cys Pro Lys Thr Lys
245 250 255

Val Leu Cys Leu Trp Arg Arg Asn Gly Ser Glu Thr Gln Leu Asn Lys
260 265 270

Phe Tyr Thr Lys Lys Cys Arg Glu Leu Tyr Tyr Cys Val Lys Asp Ser
275 280 285

Met Glu Arg Ala Ala Ala Arg Gln Gln Ser Ile Lys Pro Gly Pro Glu
290 295 300

Leu Gly Gly Glu Phe Pro Val Gln Asp Leu Lys Thr Gly Glu Gly Gly
305 310 315 320

Leu Leu Gln Val Thr Leu Glu Gly Ile Asn Leu Lys Phe Met His Asn
325 330 335

Gln Val Phe Ile Glu Leu Asn His Ile Lys Lys Cys Asn Thr Val Arg
340 345 350

Gly Val Phe Val Leu Glu Glu Phe Val Pro Glu Ile Lys Glu Val Val
355 360 365

Ser His Lys Tyr Lys Thr Pro Met Ala His Glu Ile Cys Tyr Ser Val
370 375 380

Leu Cys Leu Phe Ser Tyr Val Ala Ala Val His Ser Ser Glu Glu Asp
385 390 395 400

Leu Arg Thr Pro Pro Arg Pro Val Ser Ser
405 410

<210> SEQ ID NO 19
<211> LENGTH: 138
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<223> OTHER INFORMATION: TNF receptor death domain ligand protein comprising amino acid sequence
<300> PUBLICATION INFORMATION:
<310> PATENT DOCUMENT NUMBER: 5,849,501
<311> PATENT FILING DATE: 1995-06-19
<312> PUBLICATION DATE: 1998-12-15

<400> SEQUENCE: 19

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Glu Val Gln Asp Leu Phe Glu Ala Gln Gly Asn Asp Arg Leu Lys Leu
 1               5                  10                  15

Leu Val Leu Tyr Ser Gly Glu Asp Asp Glu Leu Leu Gln Arg Ala Ala
            20                  25                  30

Ala Gly Gly Leu Ala Met Leu Thr Ser Met Arg Pro Thr Leu Cys Ser
        35                  40                  45

Arg Ile Pro Gln Val Thr Thr His Trp Leu Glu Ile Leu Gln Ala Leu
    50                  55                  60

Leu Leu Ser Ser Asn Gln Glu Leu Gln His Arg Gly Ala Val Val Val
65                  70                  75                  80

Leu Asn Met Val Glu Ala Ser Arg Glu Ile Ala Ser Thr Leu Met Glu
                85                  90                  95

Ser Glu Met Met Glu Ile Leu Ser Val Leu Ala Lys Gly Asp His Ser
            100                 105                 110

Pro Val Thr Arg Ala Ala Ala Ala Cys Leu Asp Lys Ala Val Glu Tyr
        115                 120                 125

Gly Leu Ile Gln Pro Asn Gln Asp Gly Glu
    130                 135
```

<210> SEQ ID NO 20
<211> LENGTH: 310
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<223> OTHER INFORMATION: TNF receptor death domain ligand protein comprising amino acid sequence
<300> PUBLICATION INFORMATION:
<310> PATENT DOCUMENT NUMBER: 5,849,501
<311> PATENT FILING DATE: 1995-06-19
<312> PUBLICATION DATE: 1998-12-15

<400> SEQUENCE: 20

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Ser Leu Lys Ala Asn Ile Pro Glu Val Glu Ala Val Leu Asn Thr Asp
 1               5                  10                  15

Arg Ser Leu Val Cys Asp Gly Lys Arg Gly Leu Leu Thr Arg Leu Leu
            20                  25                  30

Gln Val Met Lys Lys Glu Pro Ala Glu Ser Ser Phe Arg Phe Trp Gln
        35                  40                  45

Ala Arg Ala Val Glu Ser Phe Leu Arg Gly Thr Thr Ser Tyr Ala Asp
    50                  55                  60

Gln Met Phe Leu Leu Lys Arg Gly Leu Leu Glu His Ile Leu Tyr Cys
65                  70                  75                  80

Ile Val Asp Ser Glu Cys Lys Ser Arg Asp Val Leu Gln Ser Tyr Phe
                85                  90                  95

Asp Leu Leu Gly Glu Leu Met Lys Phe Asn Val Asp Ala Phe Lys Arg
            100                 105                 110

Phe Asn Lys Tyr Ile Asn Thr Asp Ala Lys Phe Gln Val Phe Leu Lys
        115                 120                 125

Gln Ile Asn Ser Ser Leu Val Asp Ser Asn Met Leu Val Arg Cys Val
    130                 135                 140

Thr Leu Ser Leu Asp Arg Phe Glu Asn Gln Val Asp Met Lys Val Ala
145                 150                 155                 160

Glu Val Leu Ser Glu Cys Arg Leu Leu Ala Tyr Ile Ser Gln Val Pro
                165                 170                 175

Thr Gln Met Ser Phe Leu Phe Arg Leu Ile Asn Ile Ile His Val Gln
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-continued

180 185 190

Thr Leu Thr Gln Glu Asn Val Ser Cys Leu Asn Thr Ser Leu Val Ile
195 200 205

Leu Met Leu Ala Arg Arg Lys Glu Arg Leu Pro Leu Tyr Leu Arg Leu
210 215 220

Leu Gln Arg Met Glu His Ser Lys Lys Tyr Pro Gly Phe Leu Leu Asn
225 230 235 240

Asn Phe His Asn Leu Leu Arg Phe Trp Gln Gln His Tyr Leu His Lys
245 250 255

Asp Lys Asp Ser Thr Cys Leu Glu Asn Ser Ser Cys Ile Ser Phe Ser
260 265 270

Tyr Trp Lys Glu Thr Val Ser Ile Leu Leu Asn Pro Asp Arg Gln Ser
275 280 285

Pro Ser Ala Leu Val Ser Tyr Ile Glu Glu Pro Tyr Met Asp Ile Asp
290 295 300

Arg Asp Phe Thr Glu Glu
305 310

<210> SEQ ID NO 21
<211> LENGTH: 607
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<223> OTHER INFORMATION: TNF receptor death domain ligand protein comprising amino acid sequence
<300> PUBLICATION INFORMATION:
<310> PATENT DOCUMENT NUMBER: 5,849,501
<311> PATENT FILING DATE: 1995-06-19
<312> PUBLICATION DATE: 1998-12-15

<400> SEQUENCE: 21

Glu Ile Ser Arg Lys Val Tyr Lys Gly Met Leu Asp Leu Leu Lys Cys
1 5 10 15

Thr Val Leu Ser Leu Glu Gln Ser Tyr Ala His Ala Gly Leu Gly Gly
20 25 30

Met Ala Ser Ile Phe Gly Leu Leu Glu Ile Ala Gln Thr His Tyr Tyr
35 40 45

Ser Lys Glu Pro Asp Lys Arg Lys Arg Ser Pro Thr Glu Ser Val Asn
50 55 60

Thr Pro Val Gly Lys Asp Pro Gly Leu Ala Gly Arg Gly Asp Pro Lys
65 70 75 80

Ala Met Ala Gln Leu Arg Val Pro Gln Leu Gly Pro Arg Ala Pro Ser
85 90 95

Ala Thr Gly Lys Gly Pro Lys Glu Leu Asp Thr Arg Ser Leu Lys Glu
100 105 110

Glu Asn Phe Ile Ala Ser Ile Gly Pro Glu Val Ile Lys Pro Val Phe
115 120 125

Asp Leu Gly Glu Thr Glu Glu Lys Lys Ser Gln Ile Ser Ala Asp Ser
130 135 140

Gly Val Ser Leu Thr Ser Ser Ser Gln Arg Thr Asp Gln Asp Ser Val
145 150 155 160

Ile Gly Val Ser Pro Ala Val Met Ile Arg Ser Ser Ser Gln Asp Ser
165 170 175

Glu Val Ser Thr Val Val Ser Asn Ser Ser Gly Glu Thr Leu Gly Ala
180 185 190

-continued

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Asp Ser Asp Leu Ser Ser Asn Ala Gly Asp Gly Pro Gly Gly Glu Gly
        195                 200                 205

Ser Val His Leu Ala Ser Ser Arg Gly Thr Leu Ser Asp Ser Glu Ile
    210                 215                 220

Glu Thr Asn Ser Ala Thr Ser Thr Ile Phe Gly Lys Ala His Ser Leu
225                 230                 235                 240

Lys Pro Ser Ile Lys Glu Lys Leu Ala Gly Ser Pro Ile Arg Thr Ser
                245                 250                 255

Glu Asp Val Ser Gln Arg Val Tyr Leu Tyr Glu Gly Leu Leu Gly Lys
            260                 265                 270

Glu Arg Ser Thr Leu Trp Asp Gln Met Gln Phe Trp Glu Asp Ala Phe
        275                 280                 285

Leu Asp Ala Val Met Leu Glu Arg Glu Gly Met Gly Met Asp Gln Gly
    290                 295                 300

Pro Gln Glu Met Ile Asp Arg Tyr Leu Ser Leu Gly Glu His Asp Arg
305                 310                 315                 320

Lys Arg Leu Glu Asp Asp Glu Asp Arg Leu Leu Ala Thr Leu Leu His
                325                 330                 335

Asn Leu Ile Ser Tyr Met Leu Leu Met Lys Val Asn Lys Asn Asp Ile
            340                 345                 350

Arg Lys Lys Val Arg Arg Leu Met Gly Lys Ser His Ile Gly Leu Val
        355                 360                 365

Tyr Ser Gln Gln Ile Asn Glu Val Leu Asp Gln Leu Ala Asn Leu Asn
    370                 375                 380

Gly Arg Asp Leu Ser Ile Trp Ser Ser Gly Ser Arg His Met Lys Lys
385                 390                 395                 400

Gln Thr Phe Val Val His Ala Gly Thr Asp Thr Asn Gly Asp Ile Phe
                405                 410                 415

Phe Met Glu Val Cys Asp Asp Cys Val Val Leu Arg Ser Asn Ile Gly
            420                 425                 430

Thr Val Tyr Glu Arg Trp Trp Tyr Glu Lys Leu Ile Asn Met Thr Tyr
        435                 440                 445

Cys Pro Lys Thr Lys Val Leu Cys Leu Trp Arg Arg Asn Gly Ser Glu
    450                 455                 460

Thr Gln Leu Asn Lys Phe Tyr Thr Lys Lys Cys Arg Glu Leu Tyr Tyr
465                 470                 475                 480

Cys Val Lys Asp Ser Met Glu Arg Ala Ala Ala Arg Gln Gln Ser Ile
                485                 490                 495

Lys Pro Gly Pro Glu Leu Gly Gly Glu Phe Pro Val Gln Asp Leu Lys
            500                 505                 510

Thr Gly Glu Gly Gly Leu Leu Gln Val Thr Leu Glu Gly Ile Asn Leu
        515                 520                 525

Lys Phe Met His Asn Gln Val Phe Ile Glu Leu Asn His Ile Lys Lys
    530                 535                 540

Cys Asn Thr Val Arg Gly Val Phe Val Leu Glu Glu Phe Val Pro Glu
545                 550                 555                 560

Ile Lys Glu Val Val Ser His Lys Tyr Lys Thr Pro Met Ala His Glu
                565                 570                 575

Ile Cys Tyr Ser Val Leu Cys Leu Phe Ser Tyr Val Ala Ala Val His
            580                 585                 590
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-continued

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Ser Ser Glu Glu Asp Leu Arg Thr Pro Pro Arg Pro Val Ser Ser
        595                 600                 605
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<210> SEQ ID NO 22
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA which is antisense to TNF-alpha
<300> PUBLICATION INFORMATION:
<310> PATENT DOCUMENT NUMBER: 5,705,389
<311> PATENT FILING DATE: 1994-11-18
<312> PUBLICATION DATE: 1998-01-06

<400> SEQUENCE: 22

tcatggtgtc ctttgcag 18

<210> SEQ ID NO 23
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA upper primer for Dengue virus type 2 detection

<400> SEQUENCE: 23

aatatgctga aacgcgagag aaaccg 26

<210> SEQ ID NO 24
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA lower primer for Dengue virus type 2 detection

<400> SEQUENCE: 24

aaggaacgcc accaaggcca tg 22

<210> SEQ ID NO 25
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA upper primer for IL-1ra detection

<400> SEQUENCE: 25

cgggatccgg gagaaaatcc agcaagatg 29

<210> SEQ ID NO 26
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
DNA lower primer for IL-1ra detection

<400> SEQUENCE: 26

aggtcctgct catcccctta aggc 24

What is claimed is:

1. A method of treating a disease, condition or disorder, comprising:

administering blood or a soluble-receptor containing fraction thereof to a mammal suffering from an acute inflammatory condition, wherein prior to administration the blood or fraction thereof has been contacted with tetracycline or a tetracycline-like compound, whereby the level of a pre-selected cytokine receptor in the blood is at least three-fold higher than level of the receptors prior to contacting with the tetracycline or tetracycline-like compound.

2. The method of claim 1, wherein the cytokine receptor is a tumor necrosis factor (TNF) receptor and/or an interleukin-1 receptor (IL-1R).

3. The method of claim 1, wherein the disease, condition or disorder is selected from the group consisting of acute inflammatory conditions associated with viral hemorrhagic diseases, parasitic diseases, bacterial infections, sepsis, cachexia, autoimmune disorders, acute cardiovascular events, chronic myelogenous leukemia and transplanted bone marrow-induced graft-versus-host disease, septic shock, immune complex-induced colitis, cerebrospinal fluid inflammation, autoimmune disorders, multiple sclerosis; inflammatory responses associated with trauma; systemic inflammatory response syndrome (SIRS), adult respiratory distress syndrome (ARDS), acute liver failure, inflammatory bowel disease and Crohn's disease.

4. The method of claim 1, wherein the disease, condition or disorder is selected from viral hemorrhagic diseases and bacterial infections.

5. The method of claim 1, wherein the contacting with tetracycline or a tetracycline-like compound is effected by administering the tetracycline or tetracycline-like compound to the donor of the blood.

6. The method of claim 1, wherein the mammal has a viral hemorrhagic disease.

7. A method for treating or preventing a viral hemorrhagic disease, comprising administering an effective amount of a tetracycline or tetracycline-like compound, whereby the viral hemorrhagic disease is treated or prevented.

8. The method of claim 7, further comprising administering a blood-derived composition, wherein:

the composition is produced by

i) obtaining blood from a mammalian donor and measuring the level of a cytokine antagonist or cytokine receptor in the blood; and

ii) administering to the mammalian donor or contacting blood from the donor with a tetracycline or tetracycline-like compound(s) in an amount sufficient and for a time sufficient to result in a three-fold increase in the measured cytokine antagonist or receptor; and

the composition is administered simultaneously, subsequently or before administration of the tetracycline or tetracycline-like compound.

9. The method of claim 7, further comprising administering an anti-hemorrhagic viral treatment or agent to the mammal.

10. A combination, comprising:

a) a tetracycline compound; and

b) an anti-hemorrhagic virus treatment or agent.

11. The combination of claim 10, wherein the tetracycline compound and the anti-hemorrhagic virus agent are formulated in a single pharmaceutical composition or each formulated in a separate pharmaceutical compositions.

12. The combination of claim 10, wherein the tetracycline compound is selected from the group consisting of chlortetracycline, demeclocycline, doxycycline, methacycline, minocycline, oxytetracycline and tetracycline.

13. The combination of claim 10, wherein the hemorrhagic virus is a Bunyaviridae, a Filoviridae, a Flaviviridae, or an Arenaviridae virus.

14. The combination of claim 10, wherein the anti-hemorrhagic virus agent inhibits interleukin-1 (IL-1) and/or tumor necrosis factor (TNF).

15. The combination of claim 14, wherein the agent that inhibits IL-1 is selected from the group consisting of anti-IL-1 antibodies, anti-IL-1 receptor antibodies, IL-1 receptor antagonists, IL-1 production inhibitors, IL-1 receptor production inhibitors, and IL-1 releasing inhibitors.

16. The combination of claim 14, wherein the TNF inhibitor is selected from the group consisting of an anti-TNF antibody, an anti-TNF receptor antibody, a TNF receptor antagonist, a TNF production inhibitor, a TNF receptor production inhibitor and a TNF releasing inhibitor.

17. The combination of claim 10, wherein the anti-viral-hemorrhagic agent is selected from the group consisting of an anti-viral vaccine, an anti-viral antibody, a viral-activated immune cell and a viral-activated immune serum.

18. The method of claim 9, wherein the mammal is a human.

19. The method of claim 18, wherein the tetracycline compound is selected from the group consisting of chlortetracycline, demeclocycline, doxycycline, methacycline, minocycline, oxytetracycline and tetracycline.

20. The method of claim 9, wherein the anti-viral hemorrhagic treatment comprises administering an effective amount of an anti-viral-hemorrhagic agent.

21. The method of claim 20, wherein the tetracycline compound and the anti-viral-hemorrhagic agent are administered sequentially.

22. The method of claim 21, wherein the tetracycline compound and the anti hemorrhagic virus treatment are co-administered.

23. The method of claim 22, wherein the tetracycline compound and the anti-viral-hemorrhagic agent are administered in the same composition.

24. The method of claim 9, wherein the anti-hemorrhagic virus agent inhibits interleukin-1 (IL-1) and/or tumor necrosis factor (TNF).

25. The method of claim 24, wherein the agent that inhibits IL-1 is selected from the group consisting of anti-IL-1 antibodies, anti-IL-1 receptor antibodies, IL-1 receptor antagonists, IL-1 production inhibitors, IL-1 receptor production inhibitors, and IL-1 releasing inhibitors.

26. The method of claim 25, wherein the TNF inhibitor is selected from the group consisting of an anti-TNF antibody, an anti-TNF receptor antibody, a TNF receptor antagonist, a TNF production inhibitor, a TNF receptor production inhibitor and a TNF releasing inhibitor.

27. A kit, comprising the combination of claim 10 and instructions for administration of the components for treatment of a hemorrhagic viral infection.

28. An article of manufacture, comprising:

packaging material;

a tetracycline compound or a tetracycline-like compound(s) in an amount effective for treating a hemorrhagic viral infection; and

a label indicating that the tetracycline compound is for use in treating a hemorrhagic viral infection.

29. A method for producing a cytokine-receptor-enriched blood product, comprising:

treating blood or a fraction thereof with a tetracycline or tetracycline-like compound; and

harvesting the plasma, wherein the plasma is enriched for cytokine receptors compared to the blood prior to treatment.

30. The method of claim 29, wherein the receptors are soluble tumor necrosis factor (TNF) receptors and/or interleukin-1 (IL-1) receptors.

31. The method of claim 29, wherein the blood is contacted in vitro.

32. The method of claim 29, wherein the blood is contacted in vivo.

33. The method of claim 29, further comprising harvesting the globulin fraction.

34. A method for producing cytokine-receptor-enriched compositions, comprising:

treating white blood cells in vitro with a tetracycline or tetracycline-like compound, whereby receptor expression is induced; and

collecting extracellular medium.

35. The method of claim 34, further comprising:

fractionating the medium to collect fraction(s) that contain the receptors.

36. The method of claim 34, wherein the receptors comprise soluble tumor necrosis factor (TNF) receptors and/or interleukin-1 (IL-1) receptors.

37. The method of claim 34, further comprising isolating IL-1 and/or TNF receptors therefrom.

38. A soluble receptor-containing composition produced by the method of claim 29.

39. A soluble receptor-containing composition produced by the method of claim 34.

40. A method of treatment of a mammal having an acute inflammatory condition, disease or disorder, comprising administering the composition of claim 59.

41. The method of claim 40, wherein the acute inflammatory condition is selected from the group consisting of acute inflammatory conditions associated with viral hemorrhagic diseases, parasitic diseases, bacterial infections, sepsis, cachexia, autoimmune disorders, acute cardiovascular events, chronic myelogenous leukemia and transplanted bone marrow-induced graft-versus-host disease, septic shock, immune complex-induced colitis, cerebrospinal fluid inflammation, autoimmune disorders, multiple sclerosis; inflammatory responses associated with trauma; systemic inflammatory response syndrome (SIRS), adult respiratory distress syndrome (ARDS), acute liver failure, inflammatory bowel disease and Crohn's disease.

42. A method of treatment of a mammal having an acute inflammatory condition, disease or disorder, comprising administering the composition of claim 39.

43. The method of claim 42, wherein the acute inflammatory condition is selected from the group consisting of acute inflammatory conditions associated with viral hemorrhagic diseases, parasitic diseases, bacterial infections, sepsis, cachexia, autoimmune disorders, acute cardiovascular events, chronic myelogenous leukemia and transplanted bone marrow-induced graft-versus-host disease, septic shock, immune complex-induced colitis, cerebrospinal fluid inflammation, autoimmune disorders, multiple sclerosis; inflammatory responses associated with trauma; systemic inflammatory response syndrome (SIRS), adult respiratory distress syndrome (ARDS), acute liver failure, inflammatory bowel disease and Crohn's disease.

44. A method for treatment or prophylaxis of an inflammatory disease, comprising administering an effective amount of a tetracycline or tetracycline-like compound, whereby the disease is treated or prevented, and wherein the disease, condition or disorder is selected from the group consisting of multiple sclerosis, rheumatoid arthritis, and inflammatory responses associated with systemic inflammatory response syndrome (SIRS), adult respiratory distress syndrome (ARDS), acute liver failure, inflammatory bowel disease, polytrauma, burns, major surgery or Crohn's disease.

45. The method of claim 44, wherein the tetracycline compound is selected from the group consisting of chlortetracycline, demeclocycline, doxycycline, methacycline, minocycline, oxytetracycline and tetracycline.

* * * * *

APPENDIX A

PRIMERS DESIGNED FOR DEN-2 CLONING/SEQUENCING PROJECT:

SEQ. ID NO:	PRIMER	MER/SENSE	SEQUENCE
3	pUC/M13-P5	25/+	5'-CCCAGTCACGACGTTGTAAAACGAC-3'
4	pUC/M13-P5B	27/+	5'-GGATGTGCTGCAAGGCGATTAAGTTGG-3'
5	pUC/M13-P3	25/+	5'-TGAGCGGATAACAATTTCACACAGG-3'
6	pUC/M13-P3B	27/-	5'-GGCTTTACACTTTATGCTTCCGGCTCG-3'
7	D2-1-ECO.T7	75/+	5'-GCGGATATTG/GAATTC/TCTAGA/ AATTTAATACGACTCACTATA/ AGTTGTTAGTCTACGTGGACCGACAAAGACAG-3' (5'-Fill /EcoRI /XbaI/T7 Promoter/ 5'-end of DEN-2)
8	D2-SMT71	77/+	5'-CCAGT/GAATTC/GAGCTC/ACGCGT/ AAATTTAATACGACTCACTATA/ AGTTGTTAGTCTACGTGGACCGACAAAGACAG-3' (5'-Fill/EcoRI/SstI/MluI/T7 Promoter/ 5'-end of DEN-2)
9	D2-1	24/+	5'-AGTTGTTAGTCTACGTGGACCGAC-3'
10	D2-28	34/+	5'-GACAGATTCTTTGAGGGAGCTGAGCTCAACGTAG-3'
11	D2-134	28/+	5'-TCAATATGCTGAAACGCGAGAGAAACCG-3'
12	cD2-250	26/-	5'-GGGATTGTTAGGAAACGAAGGAACGC-3'
13	D2-274	32/+	5'-CCACCAACAGCAGGGATACTGAAAAGATGGGG-3'
14	cD2-378	25/-	5'-TGCAGATCTGCGTCTCCTATTCAAG-3'
15	D2-528	25/+	5'-CGTGAACATGTGTACCCTCATGGCC-3'
16	cD2-616	26/-	5'-TTGCACCAACAGTCAATGTCTTCAGG-3'
17	D2-616	25/+	5'-ACCAGAAGACATAGATTGTTGGTGC-3'
18	cD2-618	25/-	5'-GCACCAACAGTCTATGTCTTCTGGC-3'
19	cD2-771	25/-	5'-ATGTTTCCAGGCCCCTTCTGATGAC-3'
20	D2-847	25/+	5'-GCAGCAATCCTGGCATACACCATAG-3'
21	D2-996	27/+	5'-GGTTGACATAGTCTTAGAACATGGAAG-3'
22	cD2-996	27/-	5'-CTTCCATGTTCTAAGACTATGTCAACC-3'

SEQ. ID NO:	PRIMER	MER/SENSE	SEQUENCE
23	D2-1005	35/+	5'-GTCTTAGAACATGGAAGTTGTGTGACGACGATGGC-3'
24	D2-1141	25/+	5'-ACAACAGAATCTCGCTGCCCAACAC-3'
25	D2-1211	25/+	5'-GCAAACACTCCATGGTAGACAGAGG-3'
26	cD2-1211	25/-	5'-CCTCTGTCTACCATGGAGTGTTTGC-3'
27	cD2-1227	27/-	5'-CCACATCCATTTCCCCATCCTCTGTCT-3'
28	D2-1261	30/+	5'-GGAAAGGGAGGCATTGTGACCTGTGCTATG-3'
29	D2-1416	28/+	5'-GGAAATCAAAATAACACCACAGAGTTCC-3'
30	cD2-1503	34/-	5'-CTGCAGCAACACCATCTCATTGAAGTCGAGGCCC-3'
31	D2-1510	25/+	5'-GACTTCAATGAGATGGTGCTGCTGC-3'
32	cD2-1510	25/+	5'-GCAGCAGCACCATCTCATTGAAGTC-3'
33	D2-1546	28/+	5'-AAGCTTGGCTGGTGCACAGGCAATGGTT-3'
34	cD2-1567	27/-	5'-TGGTAACGGCAGGTCTAGGAACCATTG-3'
35	D2-1777	23/+	5'-GGACATCTCAAGTGCAGGCTGAG-3'
36	cD2-1777	23/+	5'-CTCAGCCTGCACTTGAGATGTCC-3'
37	D2-1863	27/+	5'-GAAGGAAATAGCAGAAACACAACATGG-3'
38	cD2-1888	33/-	5'-CCCTTCATATTGTACTCTGATAACTATTGTTCC-3'
39	D2-2047	32/+	5'-CCTCCATTCGGAGACAGCTACATCATCATAGG-3'
40	cD2-2047	32/-	5'-CCTATGATGATGTAGCTGTCTCCGAATGGAGG-3'
41	D2-2170	29/+	5'-ATGGCCATTTTAGGTGACACAGCCTGGGA-3'
42	cD2-2200	27/ -	5'-TGTAAACACTCCTCCCAGGGATCCAAA-3'
43	D2-2308	29/+	5'-CTCATAGGAGTCATTATCACATGGATAGG-3'
44	cD2-2504	35/-	5'-GGGGATTCTGGTTGGAACTTATATTGTTCTGTCC-3'
45	cD2-2622	30/-	5'-TGATTCAATTCTGGTGTTATTTGTTTCCAC-3'
46	D2-2702	25/+	5'-AAGGAATCATGCAGGCAGGAAAACG-3'
47	cD2-2864	22/-	5'-ACTTCCAGCGAGTTCCAAGCTC-3' A A
48	D2-2992	25/+	5'-AACAGAGCCGTCCATGCCGATATGG-3'
49	cD2-3105	22/-	5'-TCCATTGCTCCAAAGGGTGTGT-3' G
50	D2-3236	25/+	5'-AGCTTGAGATGGACTTTGATTTCTG-3'

SEQ. ID NO:	PRIMER	MER/SENSE	SEQUENCE
51	cD2-3410	22/-	5'-GGTCTGATTTCCATCCCGTACC-3'
52	D2-3621	23/+	5'-GTCCTTTAGAGACCTGGGAAGAG-3'
53	cD2-3739	25/-	5'-GTTTTCTCAAGAGTAGTCCAGCTGC-3' C
54	D2-3905	25/+	5'-ATCAATTGGCAGTGACTATCATGGC-3'
55	cD2-4002	25/-	5'-TGTTAAGAGCAGTGGAGAAACGGAC-3' A G
56	cD2-4060	25/-	5'-GATTGAGACCTTTGATCGTCAACGC-3'
57	D2-4214	25/+	5'-TGACAGGACCATTAGTGGCTGGAGG-3'
58	D2-4257	34/+	5'-CGTGCTCACTGGACGATCGGCCGATTTGGAACTG-3'
59	cD2-4323	24/-	5'-GGGCTGCTTCCTGATATTTCTGCC-3' C
60	D2-4497	25/+	5'-CCTGTGGGAAGTGAAGAAACAACGG-3'
61	cD2-4557	30/-	5'-GCTCCATCTTCCAGTTCAGCCTTTCCCATG-3'
62	cD2-4615	25/-	5'-CTCCGGCTCCAATCTGAGAGTATCC-3' G G A
63	D2-4746	25/+	5'-CCTAATATCATATGGAGGAGGCTGG-3'
64	D2-4792	25/+	5'-GAAGGAGAAGAAGTCCAGGTATTGG-3'
65	cD2-4922	25/-	5'-CTGTCGACAATTGGAGATCCTGACG-3' T T
66	D2-4994	25/+	5'-GTGGAGCATATGTGAGTGCTATAGC-3'
67	D2-5124	25/+	5'-TCTGACTATGGCCGGAAGGTATCTC-3'
68	D2-5173	25/+	5'-ACATTAATCTTGGCCCCCACTAGAG-3'
69	cD2-5272	19/-	5'-CGATCTCCCGCCCGGTGTG-3' A
70	cD2-5318	25/-	5'-CTAACTGGTGATAGCAGCCTCATGG-3'
71	cD2-5656	27/-	5'-CCTACTGAGTTGTATCACTTTCTTTCC-3'
72	cD2-5891	26/-	5'-TGGATTTCTTCCTATTCTCCCTCTTC-3'
73	D2-5770	25/+	5'-TTCAAGGCTGAGAGGGTTATAGACC-3'
74	D2-6152	25/+	5'-TCTGGTTGGCCTACAGAGTGGCAGC-3'
75	cD2-6252	27/-	5'-CCTTCTTTTGTCCAGATTTCCACTTCC-3' A

SEQ. ID NO:	PRIMER	MER/SENSE	SEQUENCE
76	D2-6493	35/+	5'-GCGTACAACCATGCTCTCAGTGAACTGCCGGAGAC-3'
77	cD2-6605	24/-	5'-TTCCCAGGGTCATCTTCCCTATAC-3' G
78	cD2-6624	31/-	5'-GATGCTAGCCGTGATTATGCAGCACATTCCC-3'
79	D2-6748	25/+	5'-AAACAGAGAACACCCCAAGACAACC-3'
80	cD2-6932	21/-	5'-CGGCATACAGCGTCCATGCTG-3'
81	D2-7055	25/+	5'-GTCTCGGGAAAGGATGGCCATTGTC-3'
82	cD2-7195	25/-	5'-CTCTGGTTGCTTTTGCTTGAAGTCC-3' A G G
83	cD2-7217	27/-	5'-CCGCCGCTGCTCTTTTCTGAGCTTCTC-3'
84	D2-7378	25/+	5'-AGGACTACATGGGCTCTGTGTGAGG-3'
85	cD2-7515	19/-	5'-GAGAAGTCCAGCTCCGGCC-3'
86	D2-7769	25/+	5'-AGAGAAACATGGTCACACCAGAAGG-3'
87	cD2-7885	22/-	5'-GTTCTTCGTGTCCTGGTCCTCC-3'
88	D2-8165	25/+	5'-GGAAATATGGAGGAGCCTAGTGAGG-3'
89	cD2-8210	22/-	5'-ACCCAGTACATCTCATGTGTGG-3'
90	D2-8428	28/+	5'-GAGCATGAAACATCATGGCACTATGACC-3'
91	D2-8440	25/+	5'-TCATGGCACTATGACCAAGACCACC-3'
92	cD2-8529	22/-	5'-CAGTCTGACCACTCCGTTCACC-3' C A G
93	D2-8773	25/+	5'-AAGGTGAGAAGCAATGCAGCCTTGG-3'
94	D2-8798	29/+	5'-GGGCCATATTCACTGATGAGAACAAGTGG-3'
95	cD2-8865	22/-	5'-TCTTTCCCTGTCAACCAGCTCC-3' C T
96	D2-9046	25/+	5'-AATGAAGATCACTGGTTCTCCAGAG-3'
97	D2-9131	25/+	5'-ACGTGAGCAAGAAAGAGGGAGGAGC-3'
98	cD2-9166	22/-	5'-TGTCCCATCCTGCTGTGTCATC-3' A G
99	cD2-9234	30/-	5'-GCTAGTTTCTTGTGTTCTCCTTCCATGTGG-3'
100	D2-9344	25/+	5'-TCATATCGAGAAGAGACCAAAGAGG-3'
101	cD2-9429	24/-	5'-ACTCCTTCTCCCTCCATCTGTCTG-3'

SEQ. ID NO:	PRIMER	MER/SENSE	SEQUENCE
102	cD2-9438	27/-	5'-ATGCTTTTGAAGATTCCTTCTCCCTCC-3' A C
103	cD2-9468	32/-	5'-GCACAGCGATTTCTTCTGTGATTGTTAGGTGC-3'
104	D2-9645	25/+	5'-ACAATGGGAACCTTCAAGAGGATGG-3'
105	D2-9656.BAM	45/+	5'-TTATCACATT/GGATCC/TTCAAGAGGATGGA ATGATTGGACACAAG-3' (5'-Fill/BamHI/DEN-2 Sequence)
106	cD2-9668	28/-	5'-CAGAAGGGCACTTGTGTCCAATCATTCC-3'
107	cD2-9779	21/-	5'-CTCCCTGGGAAATTCGGGCTC-3' T G
108	cD2-9796	28/-	5'-CCGTCTCCCGCAAAGACCACCCTGCTCC-3'
109	cD2-9796.XBA	44/-	5'-TTATCACCTA/TCTAGA/CCGTCTCCC GCAAAGACCACCCTGCTCC-3'
110	cD2-9913	26/-	5'-GTTGGAACCCAATGTGATGGTACTGC-3'
111	D2-9937	25/+	5'-ACAAGTCGAACAACCTGGTCCATAC-3'
112	cD2-9977	21/-	5'-GCATGTCTTCCGTCGTCATCC-3' T
113	cD2-10003	25/-	5'-CTTGAATCCACACCCTGTTCCAGAC-3'
114	D2-10203	25/+	5'-ATACACAGATTACATGCCATCCATG-3'
115	cD2-10261	21/-	5'-TTTTGCCTTCTACCACAGGAC-3' T A
116	D2-10289	25/-	5'-GAAACAAGGCTAGAAGTCAGGTCGG-3'
117	cD2-10337	23/-	5'-GACGGGGCTCACAGGTAGCATAG-3'
118	D2-10418	25/+	5'-GCCTGTAGCTCCACCTGAGAAGGTG-3'
119	D2-10470	25/+	5'-GGAAGCTGTACGCATGGCGTAGTGG-3'
120	cD2-10530	19/-	5'-GGGCCCCCGTTGTTGCTGC-3' A
121	cD2-10687	59/-	5'-AGAACCTGTTGATTCAACAGCACCATTCCATTTTCTG-3'
122	cD2-10687.XBA	59/-	5'-TTATCACCTA/GCATGC/TCTAGA/ AGAACCTGTTGATTCAACAGCACCATTCCATTTTCTG-3' (5'-Fill/SphI/XbaI/ 3'-End DEN-2 Sequence)
123	cD2-10687.X2	52/-	5'-TTATCACCTA/TCTAGA/ GAACCTGTTGATTCAACAGCACCATTCCATTTTCTG-3' (5'-Fill/XbaI/ 3'-End DEN-2 Sequence)

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The influence of antibody levels in dengue diagnosis by polymerase chain reaction

Suk-Yin Chan *, Ingrid Maria Kautner, Sai-Kit Lam

W.H.O. Collaborating Centre for Arbovirus Reference and Research (Dengue and Dengue Haemorrhagic Fevers), Department of Medical Microbiology, Faculty of Medicine, University of Malaya, 59100 Kuala Lumpur, Malaysia

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Abstract

The potential of RT-PCR to rapidly diagnose dengue infections from both acute and convalescent phase patients' sera was evaluated. The RNA extraction method involved binding of the viral RNA to silica particles in the presence of high concentration of guanidine thiocyanate. The protocol that was established was sensitive enough to detect 40 plaque forming units per 100 microliter of serum and results could be obtained within one day. Results from this study indicate that clinical samples should be collected in the early acute phase of illness when anti-dengue antibodies were undetectable or of low titres to ensure a more reliable diagnosis.

Keywords: Dengue diagnosis; Anti-dengue antibody; RT-PCR

1. Introduction

Dengue is considered the single most important mosquito-borne viral disease of humans in terms of morbidity and mortality. It occurs in the tropical and sub-tropical regions in which vectors, mainly *Aedes* (*Ae.*) *aegypti* and *Ae. albopictus* can be found. The illness caused by dengue viruses (family *Flaviviridae*) ranges from asymptomatic infection to undifferentiated fever, benign dengue fever, and the more severe dengue haemorrhagic fever with or without shock. Death from shock can be averted by early intervention.

* Corresponding author.

Currently, the laboratory diagnosis of dengue infection depends upon isolation of dengue virus from infected mosquito cell cultures (Tesh, 1979), inoculation of adult mosquitoes (Kuberski and Rosen, 1977) or mosquito larvae (Lam et al., 1986) and detection of anti-dengue IgM antibodies and/or haemagglutination inhibition (HI) antibodies. However, virus isolation is tedious and time consuming and the serological tests are often not sufficiently specific and sensitive.

The polymerase chain reaction (PCR) (Saiki et al., 1985; Mullis and Faloona, 1987) has the potential for sensitive, specific and rapid detection of minute quantities of genetic material in patients' serum samples. Using PCR, dengue virus has been detected directly in patients' sera (Deubel et al., 1990; Lanciotti et al., 1992) and in dengue-infected mosquito cell culture supernatant (Morita et al., 1991). The potential of PCR as a rapid and reliable diagnostic tool for dengue infections in serum samples with varying levels of specific antibodies was evaluated in this study.

2. Materials and methods

2.1. Dengue virus strains

Dengue 2 (New Guinea C) and Dengue 3 (sample 03472, isolated from the serum of a dengue fever patient) strains were used as reference strains in this study. DEN-2 and DEN-3 were selected for evaluation because they were the predominant serotypes during the study period. The serotypes were confirmed by immunofluorescence (Henchal et al., 1982) using dengue serotype-specific monoclonal antibodies, WRAIR-5-1F1 (anti-dengue 1), WRAIR-2-3H5 (anti-dengue 2), WRAIR-6-8A1 (anti-dengue 3), and WRAIR-4-1H10 (anti-dengue 4) obtained from the Walter Reed Army Institute of Research, USA.

2.2. Cell culture

A mosquito cell line from *Ae. albopictus* clone C6/36 (Igarashi, 1978) was used for propagation and isolation of dengue viruses from serum samples. Plaque assay of the virus was performed using porcine spleen cell line grown in Leibovitz-15 maintenance medium at 37°C (Russell and Nisalak, 1967).

2.3. Serum samples

Serum samples were obtained from patients with virologically and/or serologically confirmed dengue infection. Virological confirmation was by isolation of dengue viruses in C6/36 cells or mosquito larvae and serotype identification as described above. Dengue infection was serologically diagnosed by demonstration of anti-dengue IgM (Lam et al., 1987) and/or increasing HI antibodies in paired sera (Clarke and Casals, 1958).

The following groups of sera were tested:

Group 1 consisted of 26 virologically positive serum samples.

Group 2 consisted of 6 single serum samples from dengue patients with anti-dengue IgM and high titres of HI antibodies (at least 1:1280).

Group 3 consisted of 25 paired sera from dengue patients with four-fold or greater rise in titres of HI antibodies, 14 as a result of primary infection and 11 as a result of secondary infection (W.H.O., 1986).

Group 4 consisted of 6 single serum samples from patients not suspected of dengue infection, with no anti-dengue antibodies and no dengue virus detected.

2.4. Dengue viral RNA extraction

A protocol modified from that described by Boom et al. (1990), was used to extract and purify dengue viral RNA from serum samples. Between 50 and 100 μl of patient's serum was added to a mixture of 30 μl of size-fractionated silica particles and 200 μl of lysis buffer L6 (8 M guanidine thiocyanate, 0.1 M Tris-HCl (pH 6.4)), 36 mM EDTA (pH 8.0), 0.2% Triton X-100) in a 1.5 ml Eppendorf tube. The mixture was vortexed and left at room temperature for 10 min, after which it was centrifuged at 13000 r.p.m. for about 30 s to sediment the nucleic acid-silica particle complexes. The pellet was washed once with 200 μl of washing buffer L2 (10 M guanidine thiocyanate, 0.1 M Tris-HCl (pH 6.4) twice with 500 μl of 70% (v/v) ethanol and once with 500 μl of acetone.The pellet was dried at 56°C for 10 min and the nucleic acids eluted with 50 μl of TE (10 mM Tris-HCl, 1 mM EDTA) buffer (pH 8.0), also at 56°C for 10 min. The tube was centrifuged at 13000 r.p.m. for 2 min to sediment the silica particles and 14.5 μl of the supernatant containing the RNA was used for cDNA synthesis and enzymatic DNA amplification.

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

The RNA was first heat denatured and reverse transcribed in a final volume of 20 μl of PCR buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.0), 2.0 mM $MgCl_2$, 0.5 mM each of the four deoxynucleoside triphosphates, 100 ng random primers, 15 units RNasin (Promega) and 5 units AMV RT (Stratagene). The reaction was incubated at 42°C for 60 min. PCR was carried out using one third of the cDNA. Two separate PCR reactions were set up for each sample with 50 pmol each of DEN-2 and DEN-3 serotype specific primers that annealed specifically to regions of the envelope protein gene sequences of the respective serotypes (Deubel et al., 1990). The reaction volume was adjusted to 50 μl with the final concentration of deoxynucleotides being 0.2 mM. The mixture was heat denatured and quick chilled on ice. Two units of *Taq* DNA polymerase (Stratagene) and 35 μl of liquid paraffin were added and the DNA amplified through 35 cycles of denaturation at 94°C, primer annealing at 55°C and extension at 72°C for 1 min each. After the last cycle, samples were kept at 72°C for 12 min.

2.6. Detection of RT-PCR products

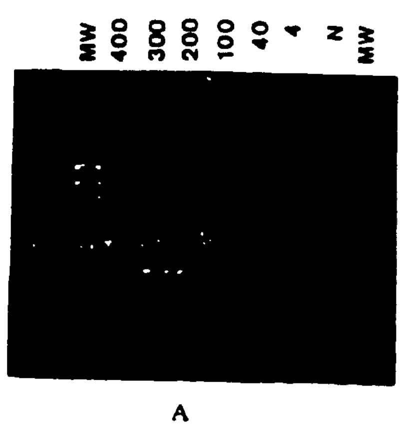
An aliquot of 5 μl of the RT-PCR products was analysed by agarose gel electrophoresis. The size of the RT-PCR products which resulted from the amplification of DEN-2 and DEN-3 was 266 bp and 257 bp, respectively. Alkaline transfer of DNA from agarose gels under vacuum was then performed. Alternatively, an aliquot of the RT-PCR

products was heat denatured at 95°C for 10 min and then spotted in duplicate onto untreated nylon membranes. The membranes were dried and the DNA fixed by UV crosslinking for 3 min. Prehybridization was carried out at 68°C for at least 1 h in a buffer containing 0.3% skim milk, 6 × SSC (sodium chloride-sodium citrate), 0.1% SDS (sodium dodecyl sulphate). Overnight hybridization was carried out at 68°C in the same buffer containing the freshly denatured digoxigenin-11-dUTP labelled dengue serotype specific cloned probe (Deubel et al., 1990) at a concentration of 100 ng/ml. The membranes were washed and processed according to the procedures described by Boehringer Mannheim.

3. Results

In order to test the sensitivity of the method, known titres of dengue serotype 2 (New Guinea C) virus were diluted in 100 μl of pooled normal human serum and RT-PCR performed. The virus titres ranged from 400, 300, 200, 100, 40, and 4 plaque forming units (PFU) per reaction tube. A negative control was also included. Agarose gel analysis in Fig. 1A shows that at least 40 PFU of DEN-2 (New Guinea C) could be detected in 100 μl of serum. As expected, no RT-PCR product corresponding to the expected size was observed in the negative control. To further verify the specificity and sensitivity of the method, hybridization using a digoxigenin-labelled DEN-2 probe was performed. Hybridization detected 4 PFU per 100 μl of serum (Fig. 1B).

All 26 serum samples from which dengue viruses were isolated (Group 1; 7 DEN-2 and 19 DEN-3) were correctly identified by RT-PCR and hybridization. These sera were



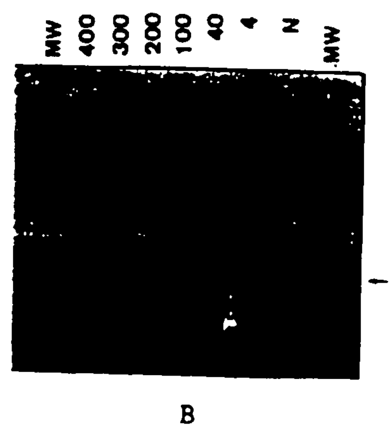


Fig. 1. Sensitivity of RT-PCR in the detection of DEN-2 (New Guinea C) virus in normal human serum. (A) Composite agarose gel electrophoresis (3% NuSieve and 1% agarose); (B) hybridization with serotype specific cloned probe. The number of DEN-2 viral particles (PFU) is indicated above each lane. Lane marked MW contains *Hin*cII-digested PhiX-174 as molecular weight markers and N, the negative control. Arrow indicates the amplified DNA fragment.

Table 1
Comparison of HI, IgM capture ELISA and RT-PCR using acute and convalescent phase serum

Sample	HI positive	IgM positive	Virus isolation	RT-PCR positive
Acute	*	3/25	10/21	25/25
Convalescent	25/25	23/25	0/16	2/25

* Paired samples are required to indicate a rise in titre of diagnostic value

from patients with dengue fever and collected within 6 days of onset of illness. Twelve of these samples had detectable level of HI antibodies of less than 1:80 and anti-dengue IgM was also detected in 4 of them. Of the 6 single serum samples (Group 2) with high titres of anti-dengue HI and IgM antibodies, only 1 was identified as DEN-3 by RT-PCR. No dengue virus was isolated from these 6 samples in cell culture.

The 6 negative controls (Group 4) were found to be negative by RT-PCR and hybridization.

Twenty-five paired serum samples were compared to determine the effect of time of collection on the outcome of RT-PCR. All of these paired samples were found positive by the HI test. Sixteen of the acute phase sera were collected within 6 days of onset of illness. The day of collection for the remaining samples was not available. All acute phase samples had low titres of HI antibodies (less than 1:160). Anti-dengue IgM was detected in 3 of the 25 acute phase samples and in 23 of the convalescent phase samples. Only 10 of the 21 acute phase samples tested were found positive by virus isolation. Dengue viruses were not isolated from the convalescent phase samples. All 25 acute phase serum samples were shown to be RT-PCR positive (Table 1). On the other hand,

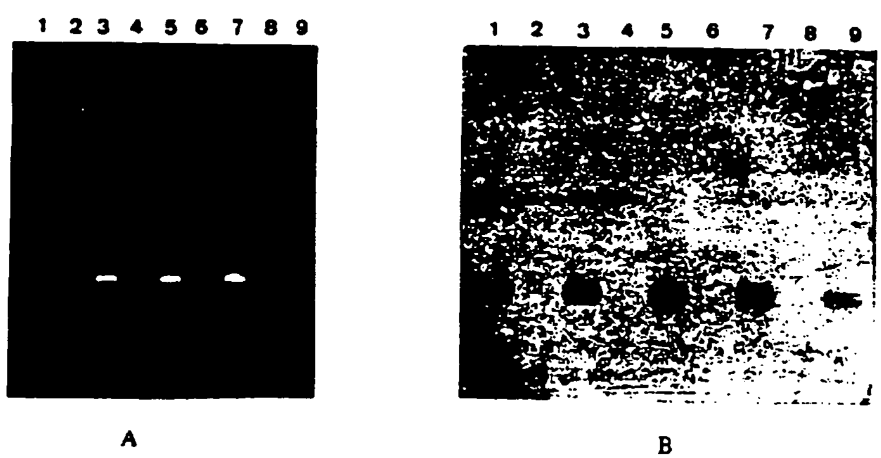


Fig. 2. RT-PCR in the detection of DEN-3 viral RNA in acute and convalescent samples. (A) Agarose gel electrophoresis; (B) Southern-blot hybridization, membrane was hybridized with probes specific for DEN-3. Lanes: (1) 03024 (a), (2) 03024 (c); (3) 01859 (a), (4) 01859 (c); (5) 03422 (a), (6) 03422 (c); (7) 02513 (a), (8) 02513 (c); (9) 03472, positive control (a): acute phase; (c): convalescent phase.

only 2 convalescent phase samples were positive by RT-PCR. Hybridization test, which was shown to be ten times more sensitive than agarose gel analysis, failed to detect any dengue-derived amplified DNA in the convalescent phase samples. Agarose gel analysis and hybridization of some representative samples are shown in Fig. 2.

4. Discussion

RT-PCR was found to be more sensitive than virus isolation, IgM capture ELISA and HI tests in the early diagnosis of dengue infection. All of the 26 virologically positive serum samples were correctly identified by RT-PCR, thus giving a 100% specificity and sensitivity correlation with virus isolation results. The 6 negative control sera which were negative for dengue infection by virus isolation, anti-dengue IgM, and HI antibodies, remained negative by RT-PCR. This shows that the primers used are specific only towards the detection of dengue derived cDNA. Of the 25 serologically confirmed paired serum samples tested, all of the acute phase samples from both primary and secondary dengue infections were found positive by RT-PCR compared to 12% by IgM capture ELISA and 48% by virus isolation. None of the acute phase samples were found positive for HI antibodies but with the availability of convalescent phase samples, all 25 samples showed a four-fold or greater rise in titre needed to be of diagnostic value for a recent dengue infection.

This study indicates that RT-PCR was most useful for diagnosing dengue infection in acute phase sera when anti-dengue antibodies were low or undetectable. It appears that viraemia declines with the appearance of anti-dengue antibodies. This is consistent with observations made by Innis et al. (1989), Gubler et al. (1979, 1981) and Nogueira et al. (1992) that there was an inverse correlation between virus isolation and anti-dengue antibodies. Therefore, samples for RT-PCR should be collected in the early phase of illness to ensure a more reliable diagnosis.

Although hybridization was ten times more sensitive, the overall results of RT-PCR and analysis using agarose gel electrophoresis compared favourably with hybridization. Since the detection limit of 400 PFU per ml is well below previously reported virus titres in serum of infected humans, that is between 10^3 and 10^6 human infectious doses of virus per ml (Sabin, 1952; Gubler et al., 1978), this RT-PCR method should be sensitive enough to detect dengue virus in viraemic serum samples.

RT-PCR can play a role in diagnosing dengue infection in cases where the current laboratory tests fail to give conclusive results or when a rapid test is needed to confirm the clinician's diagnosis to enable better patient management. It is also potentially useful in determining the predominant serotype for the season. This can serve as an indication of disease severity since certain serotypes are associated with more severe clinical disease (Lam and George, 1991). In addition, this technique will now be applied to the examination of peripheral blood leukocytes obtained from dengue-infected patients, and the results compared to serum samples from both the acute and convalescent phases. This approach may be especially valuable in diagnosing dengue at a later stage of the infection.

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Prognosis in HIV-1 Infection Predicted by the Quantity of Virus in Plasma

John W. Mellors,* Charles R. Rinaldo Jr., Phalguni Gupta, Roseanne M. White, John A. Todd, Lawrence A. Kingsley

The relation between viremia and clinical outcome in individuals infected with human immunodeficiency virus–type 1 (HIV-1) has important implications for therapeutic research and clinical care. HIV-1 RNA in plasma was quantified with a branched-DNA signal amplification assay as a measure of viral load in a cohort of 180 seropositive men studied for more than 10 years. The risk of acquired immunodeficiency syndrome (AIDS) and death in study subjects, including those with normal numbers of CD4⁺ T cells, was directly related to plasma viral load at study entry. Plasma viral load was a better predictor of progression to AIDS and death than was the number of CD4⁺ T cells.

The prognosis of individuals infected with HIV-1 is variable. In adults, the average time between infection and development of AIDS is 10 to 11 years (*1*), but a significant proportion of individuals (~20%) progresses rapidly to AIDS within 5 years of infection (*2*). At the other extreme, it is estimated that 12% of infected individuals will remain free of AIDS for 20 years (*2*).

Many clinical and laboratory markers have been used to estimate prognosis in HIV-1 infection (*3*). Although the single best predictor of AIDS onset characterized to date is the percentage or absolute number of circulating CD4⁺ T cells (*4*), a marker that could be used to assess risk before substantial immune destruction has occurred would be preferable. Recent interest has focused on measurement of HIV-1 RNA in cells or plasma for prediction of outcome (*5, 6*). Previous studies have suggested that the amount of HIV-1 RNA in plasma soon after HIV-1 infection (seroconversion) is a good CD4⁺ T cell–independent predictor of AIDS risk (*7*). In clinical settings, however, the date and duration of HIV-1 infection are usually not known. We have now investigated the prognostic value of plasma HIV-1 RNA measurements in a large cohort of HIV-1–infected men for whom the duration of infection at study entry was not known.

The study population consisted of 209 HIV-1–infected gay or bisexual men enrolled in the Pittsburgh portion of the Multicenter AIDS Cohort Study (MACS) between April 1984 and March 1985 (*8, 9*). These 209 men constitute all of the HIV-1–seropositive men enrolled at the Pittsburgh site; thus, there was no selection bias in choosing the study population. Clinical status, CD4⁺ T cell counts (*10, 11*), and specimens for laboratory studies were obtained at study entry (baseline) and at follow-up visits every 6 months. Heparinized plasma samples for HIV-1 RNA quantification were processed within 2 to 20 hours of collection and stored at −70°C until testing (*12*). Samples from 180 (86%) of the 209 men were available for testing. HIV-1 RNA was measured with an ultrasensitive branched-DNA signal amplification assay, which has a quantification limit of 500 molecules/ml and a linear dynamic range of up to 1.6×10^6 molecules/ml (*13, 14*).

Subjects were followed for progression to AIDS (1987 Centers for Disease Control definition) and death. Median follow-up was 5.6 years (range, 0.02 to 10.6 years) for those who developed AIDS, and 10.6 years (range, 3.2 to 11.2 years) for those who remained free of AIDS. None of the subjects had received antiretroviral therapy by study entry or by the 6-month follow-up visit, and only 74 (41%) received antiretroviral therapy at any time during follow-up.

HIV-1 RNA concentrations in plasma samples obtained at study entry (baseline) were normally distributed over a range of <500 to 294,200 molecules/ml (Fig. 1A). In only 11 (6.1%) of 180 samples were baseline HIV-1 RNA concentrations below ...

... counts, although the association was weak (Spearman's $r = -0.27$) (Fig. 1B). For example, among individuals with 400 to 800 CD4⁺ T cells/μl, there was an ~400-fold range in HIV-1 RNA concentrations (≤500 to 192,200 molecules/ml). Thus, the CD4⁺ T cell count in a subject within any CD4⁺ T cell range was a grossly inaccurate indicator of the level of viremia.

The relations between baseline viral load or baseline CD4⁺ T cell count and progression to AIDS or death were examined with Kaplan-Meier survival curves (*15*). Kaplan-Meier estimates of the proportion of subjects who progressed to either AIDS or death, stratified by quartiles according to baseline HIV-1 RNA concentrations or CD4⁺ T cell counts, revealed that baseline viral load provided excellent discrimination of both time to AIDS and time to death (Mantel-Haenszel test, $P < 0.001$) (Fig. 2). For quartiles ranging from the lowest through the highest viral load, the proportion of subjects who progressed to AIDS by 5 years after study entry were 8, 26, 49, and 62%, respectively (Fig. 2A). The median times to development of AIDS for subjects in these viral load quartiles were >10, 7.7, 5.3, and 3.5 years, respectively.

Because death from AIDS was observed a median of 15 months after AIDS diagnosis, the Kaplan-Meier estimates of time to death were greater than those for the time to AIDS. For quartiles ranging from the lowest through the highest viral load, the proportions of subjects who died within 5 years were 5, 10, 25, and 49%, respectively (Fig. 2B). The median estimated survival times in these viral load quartiles were >10, 9.5, 7.4, and 5.1 years, respectively. In contrast to the close relation between baseline viral load and outcome, baseline CD4⁺ T cell counts failed to show a strong gradient among quartiles for risk of AIDS or death. Among the three quartiles with the highest CD4⁺ T cell counts, no differences were observed for either time to development of AIDS (Fig. 2C) or time to death (Fig. 2D). Only the quartile of subjects with the lowest CD4⁺ T cell counts (≤321 cells/μl) was associated with a shorter time to development of AIDS or death ($P = 0.002$).

Additional evidence of the independence of viral load from CD4⁺ T cell counts in influencing prognosis is shown in Fig. 3. Among subjects with baseline CD4⁺ T cell counts of ≥500 cells/μl, there was a significant difference ($P < 0.001$) in time to death dependent on whether the baseline HIV-1 RNA concentration was greater than or less than the median value of 10,190 molecules/ml (Fig. 3A). The median time to death of ...

... not be accurately estimated because ...

J. W. Mellors, Department of Medicine, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15213, USA; Department of Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA 15261, USA; and Veterans Affairs Medical Center, Pittsburgh, PA 15240, USA.
C. R. Rinaldo Jr., Department of Pathology, School of Medicine, and Department of Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA 15261, USA.
P. Gupta, Department of Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA 15261, USA.
R. M. White and J. A. Todd, Chiron Corporation, Em-...

*To whom correspondence should be addressed at University of Pittsburgh Graduate School of Public Health, 403 Parran Hall, Pittsburgh, PA 15261, USA.

30% had died within 10 years. The major difference in outcome was evident despite the fact that the median CD4⁺ T cell count for both groups was ~780 cells/μl. Similarly, among subjects with baseline CD4⁺ T cell counts of <500 cells/μl, a significantly ($P <$ 0.001) shorter survival time was evident among subjects with a baseline HIV-1 RNA concentration greater than the median value (17,320 molecules/ml), again despite similar baseline CD4⁺ T cell counts (Fig. 3B).

To assess whether two consecutive measurements of viral load or CD4⁺ T cell counts provided more prognostic information than did single determinations, we compared the mean results of the first two study samples (obtained at study entry and the 6-month follow-up visit) and ranked them by quartile (Fig. 4). Outcome discrimination was not improved by stratifying survival curves according to mean CD4⁺ T cell counts (Fig. 4B), as compared to single baseline CD4⁺ T cell counts (Fig. 2D). In contrast, a clearer separation was obtained when survival curves were stratified according to mean HIV-1 RNA concentrations (Fig. 4A), as compared with single baseline HIV-1 RNA values (Fig. 2B). Among subjects in the highest viral load quartile, a shorter time to death was evident with mean HIV-1 RNA measurements (median survival, 3.9 years) than with single baseline HIV-1 RNA measurements (median survival, 5.1 years). These results indicate a poorer prognosis for subjects with persistently high viral load.

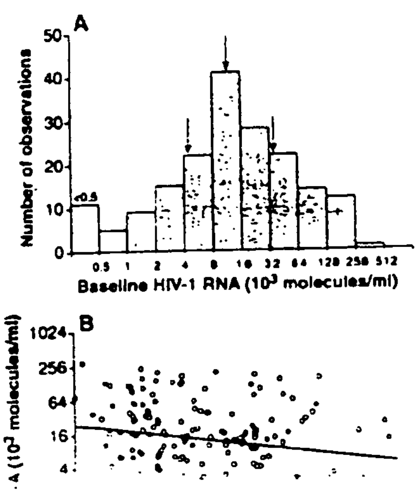
The prognosis for subjects with persistently high viral load was explored further by excluding from the analysis those subjects in whom HIV-1 RNA concentrations decreased by >80% from baseline values by the 18- or 24-month follow-up visit (Fig. 4, C and D). These subjects (n = 29) were considered to be in the recovery phase of initial HIV-1 infection at study entry because viral load had not yet fallen to a post-serconversion nadir. Among subjects in the highest viral load quartile, this analysis reduced the estimated proportion with 10-year survival from 20 to ≤5% and reduced the median survival time from 5.1 to 2.5 years (Figs. 2B and 4C). In contrast, exclusion of these 29 subjects had no effect on survival when stratified by baseline CD4⁺ T cell counts (Fig. 4D). These results provide additional evidence that persistently high viral load is almost always associated with more rapid disease progression.

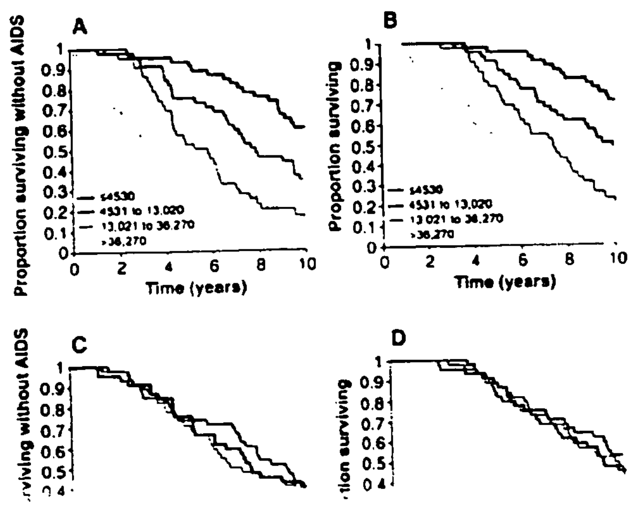
The independent effects of CD4⁺ T cell count and viral load on survival were formally examined with Cox proportional hazard models (Table 1) (16). The adjusted relative hazard of death was 1.55 ($P <$ 0.001) for each threefold increase in baseline HIV-1 RNA concentration, and 1.03 ($P >$ 0.05) for each decrease of 100 cells/μl in CD4⁺ T cell count. The unadjusted relative hazard (1.06) associated with a 100-cell decrease in CD4⁺ T cell count was statistically significant ($P <$ 0.05), but it failed to remain so after controlling for HIV-1 RNA concentration. Hence, in comparing two individuals whose T cell counts differ by 100 cells/μl at baseline, these results indicate that the individual with a threefold higher HIV-1 RNA concentration faces a 1.5-fold greater risk of death within 10 years. The risk of death was assessed as a function of changes over time in both viral load and CD4⁺ T cell count with time-dependent Cox proportional hazard analysis (Table 1). Consistent with the time-fixed analysis, the time-dependent analysis showed the adjusted relative hazard of death was 1.57 ($P <$ 0.001) for each threefold increase in HIV-1 RNA concentration from the baseline value. In this instance, however, the relative hazard of death associated with a 100-cell decrease in CD4⁺ T cell count from baseline remained statistically significant even after controlling for HIV-1 RNA values, albeit at a lower hazard (1.33).

Because the follow-up of subjects in our study was longer than that for any previous study of viral load, an analysis of long-term outcome of HIV-1 infection was possible. Such analysis showed a marked gradient of risk of disease progression and death that was directly related to the initial quantity of virus in plasma. Our observation that this risk gradient was still evident 10 years after the baseline determination emphasizes the critical influence of viral load on the course of HIV-1 infection. The Kaplan-Meier survival curves stratified by HIV-1 RNA quartile (Figs 2 and 4) show prognostic discrimination similar to that of surgical staging procedures for cancer, including traditional staging systems for Hodgkin's disease (17) and the Duke classification for colorectal carcinoma (18), both of which are based on anatomic and pathological assessments of the extent of cancer invasion and spread. For HIV-1 infection, a similar degree of prognostic discrimination can be determined with a simple test on plasma.

Initial cross-sectional analyses of HIV-1–infected individuals showed a direct relation between the extent of immunodeficiency and viral load, measured as infec-

Fig. 1. (left) (A) Frequency distribution of HIV-1 RNA concentrations ($\log_2$ scale) in baseline plasma samples. Arrows indicate location of 25th, 50th, and 75th percentiles. (B) Relation between baseline CD4⁺ T cell counts and plasma HIV-1 RNA concentration ($\log_2$ scale). The linear regression line is shown (Spearman's r = −0.27; P < 0.001). **Fig. 2.** (right) Relations between baseline markers and prognosis. Kaplan-Meier curves for AIDS-free survival (A) and survival (B) stratified by baseline HIV-1 RNA





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tious virus or viral nucleic acid (19 ... ud-ies of HIV-1 seroconverters established that the pattern of viremia after initial infection was predictive of clinical outcome (7). Individuals with persistently high viremia after seroconversion were at markedly increased risk of AIDS development (7). Saksela *et al.* (5) showed that the amount of intracellular HIV-1 mRNA in peripheral blood mononuclear cells of seroprevalent HIV-1–infected men predicted the likelihood of progression to AIDS even in individuals with >600 CD4$^+$ T cells/μl. Our study confirms and extends these observations by examining the risk of AIDS and death associated with the level of plasma viremia over a 10-year period.

In clinical practice, a CD4$^+$ T cell count of <500 cells/μl is commonly used as a trigger to initiate antiretroviral therapy. This practice requires reevaluation in light of our observations that subjects with CD4$^+$ T cell counts of ≥500 cells/μl can progress as rapidly to AIDS and death as those with much lower counts, depending on the extent of viremia. Specifically, 50% of individuals in our study with ≥500 CD4$^+$ T cells/μl (median, 780 cells/μl) and >10,900 HIV-1 RNA molecules/ml ...died within 6 years of study entry, compared with only 5% with similar CD4$^+$ T cell counts and HIV-1 RNA concentrations of ≤10,900 molecules/ml. Indeed, the rate of disease progression in subjects with ≥500 CD4$^+$ T cells/μl may exceed that in subjects with <500 cells/μl depending on the plasma viral load. Thus, treatment strategies should not be based solely on CD4$^+$ T cell numbers.

Studies by Wei *et al.* (20) and Ho *et al.* (21) have shown that viremia in HIV-1 infection is sustained by rapid, high-level viral replication, requiring continuous reinfection and destruction of CD4$^+$ T cells. Further kinetic analyses (22) indicate that the rate constant of viral clearance does not vary according to disease stage; thus, the extent of viremia depends on the rate of virus production. The factors that influence virus production in an individual are ill defined, but our study shows that a higher ... of viremia—and, by inference, greater virus production—is associated with a poorer prognosis. Higher virus production may result in more rapid exhaustion of the capacity of the immune system to replenish CD4$^+$ T cells.

Several recent studies have examined whether reductions in viral load, measured as HIV-1 RNA, in response to antiretroviral therapy correlate with delayed disease progression to AIDS and death. O'Brien *et al.* (23) have shown that reductions in plasma HIV-1 RNA concentration in response to zidovudine treatment account for a substantial portion (59%) of the benefit of this drug in delaying the onset of AIDS. A decrease in HIV-1 RNA was a better predictor of outcome than was an increase in the CD4$^+$ T cell count. Two other studies of combination drug therapy have also demonstrated that 0.5 to 1.0 $\log_{10}$ reductions in HIV-1 RNA in response to treatment cor-

Table 1. Time-fixed and time-dependent Cox proportional hazards models of survival. Each analysis shows the relative risk of death associated with a 1-unit change in the covariates (a threefold increase in HIV-1 RNA concentration and a decrease of 100 cells/μl in CD4$^+$ T cell count). The time-fixed covariate model uses only the baseline marker measurements (n = 180). The time-dependent covariate model uses all available marker measurements (total number of measurements, 984; average number of measurements per subject, 5.5).

Variable	Relative hazard of death (95% confidence intervals)			
	Time-fixed covariates		Time-dependent covariates	
	Unadjusted	Adjusted	Unadjusted	Adjusted
HIV-1 RNA	1.50*	1.55*	1.95*	1.57*
(threefold increase)	(1.36–1.82)	(1.34–1.80)	(1.72–2.21)	(1.34–1.82)
CD4$^+$ T cell count	1.06†	1.03	1.62*	1.33*
(100 cell/μl decrease)	(1.00–1.12)	(0.96–1.09)	(1.45–1.80)	(1.18–1.50)

*P < 0.001. †P < 0.05.

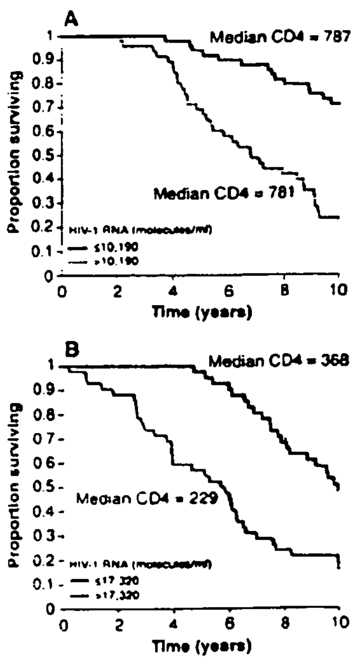

Fig. 3. Independence of the relation between prognosis and baseline HIV-1 RNA of the baseline CD4$^+$ T cell count. Kaplan-Meier survival ...

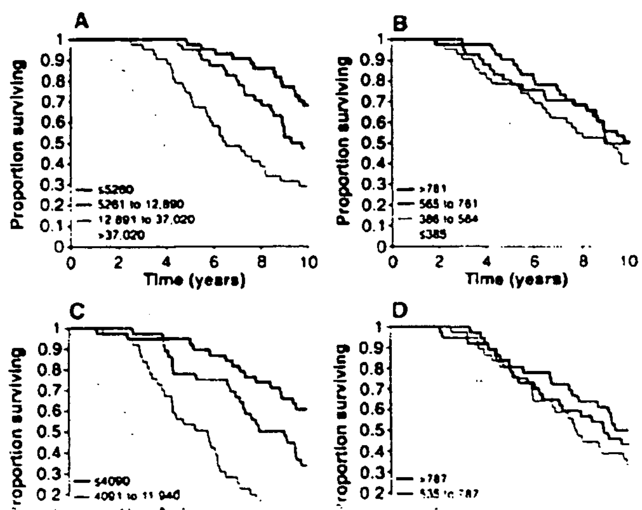

Fig. 4. (**A** and **B**) Kaplan-Meier survival curves stratified by quartiles of the mean of the first two HIV-1 RNA measurements (molecules per milliliter) (A) or CD4$^+$ T cell counts (cells per microliter) (B). Survival time for each of the 172 subjects for whom two consecutive samples were available was calculated from the number of years from the midpoint between the two viral measurements and the time of death. (**C** and **D**) Survival curves excluding subjects with a >80% decrease in ... RNA ... baseline CD4 ...

relate with delayed disease progression and death (*24*).

Collectively, these data indicate that the extent of viremia, measured as HIV-1 RNA, is the best available surrogate marker of HIV-1 disease progression. Several of the rational criteria for demonstrating the adequacy of a surrogate marker as put forward by DeGruttola *et al.* (*25*) appear to have been met: (i) Base-line HIV-1 RNA concentrations are highly predictive of prognosis. (ii) There is a strong time-dependent prognostic relation between HIV-1 RNA and outcome. And (iii) reduced concentrations of HIV-1 RNA, in response to antiretroviral therapy, are predictive of improved prognosis (*24*). Use of HIV-1 RNA as a surrogate marker should help guide future therapeutic research and individual patient management.

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13. Developed by Chiron, the assay measures HIV-1 RNA associated with viral particles that are separated from 1.0-ml plasma samples by centrifugation at 23,500*g* for 1 hour at 4°C. Performance characteristics of the assay have been described elsewhere (*14*). For our study, the interassay coefficient of variation for the positive control samples tested with each batch of experimental samples was 18%. All samples were coded and the assay operators were blinded to clinical outcomes.
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Rapid, single-step RT-PCR typing of dengue viruses using five NS3 gene primers

C.L.K. Seah [a], V.T.K. Chow [a,*], H.C. Tan [a], Y.C. Chan [a,b]

[a] *Department of Microbiology, Faculty of Medicine, National University of Singapore, Lower Kent Ridge Road, Singapore 0511*

[b] *Institute of Molecular and Cell Biology, National University of Singapore, Singapore*

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Abstract

In order to detect and type dengue viruses in serum specimens, four type-specific downstream primers were designed for use with a consensus upstream primer in a reverse transcription and polymerase chain reaction (RT-PCR) assay. RT-PCR using these five primers amplified NS3 gene fragments of diagnostic sizes of 169, 362, 265 and 426 base pairs for dengue virus types 1, 2, 3 and 4, respectively, but not for Japanese encephalitis, Kunjin and yellow fever viruses. The conventional two-step RT-PCR procedure was simplified by combining RT and PCR in a single-step format with a "hot start". This RT-PCR protocol was applied successfully to dengue virus-spiked serum and dengue patient serum samples, and could detect as few as one PFU of dengue virus. This assay offers a rapid, specific and sensitive molecular technique for the simultaneous detection and typing of dengue viruses.

Keywords: Dengue virus; Typing; Single-step RT-PCR; NS3 gene

1. Introduction

Dengue viruses are divided into four distinct serotypes (1–4) and are members of the family Flaviviridae. Their genomes consist of positive-sense RNA molecules of approx. 11 kb. They are arthropod-borne viruses and are transmitted between humans principally by the mosquito vector, *Aedes aegypti*. Infection can give rise to a wide spectrum of disease manifestations ranging from a mild, self-limiting febrile illness to more severe

* Corresponding author.

vascular and haemostatic abnormalities known as dengue haemorrhagic fever-dengue shock syndrome (World Health Organization, 1986).

Routine laboratory diagnosis often involves the detection of antibodies against dengue virus by the haemagglutination inhibition (HI) assay or IgM ELISA. These assays are neither rapid nor easy to manipulate, and they do not identify the serotype responsible for the infection owing to the high cross-seroreactivity between dengue viruses. The conventional method to determine the infecting serotype is by virus isolation in cell culture or mosquito, followed by immunofluorescent staining with dengue type-specific monoclonal antibodies. However, virus isolation takes from days to weeks and the success rate is often low because of factors such as inappropriate handling of specimens, fonnation of virus-antibody complexes and low numbers of viable virus (Monath, 1990).

The polymerase chain reaction (PCR) technique has been widely applied for the detection of many infectious disease agents including dengue viruses. Assays reported by other laboratories have utilized either four specific primer pairs, or a pair of consensus primers for a first round of amplification followed further by either hybridization with type-specific probes or nested PCR with type-specific primers for identification of the infecting dengue serotype (Deubel et al., 1990; Henchal et al., 1991; Morita et al., 1991; Lanciotti et al., 1992; Tanaka, 1993). We have devised a rapid and simple five-primer reverse transcription-polymerase chain reaction (RT-PCR) assay for the simultaneous detection and typing of dengue virus infections. Using a previously published consensus upstream primer (Chow et al., 1993) together with four newly designed type-specific downstream primers (all within the NS3 gene) in a single-step RT-PCR assay, a band of diagnostic size was amplified for each serotype.

2. Materials and methods

2.1. Virus strains

Dengue type 1 (Hawaii and S275/90), type 2 (New Guinea C), type 3 (H87), and type 4 (H241), Japanese encephalitis (Nakayama), Kunjin (MRM61C) and yellow fever (17D vaccine) viruses were used in this study. Viruses were propagated either in the C6/36 mosquito cell line maintained with Eagle's minimum essential medium (MEM) supplemented with 2% foetal calf serum and 0.1% BSA, or in Vero cells maintained with MEM supplemented with 0.1% BSA.

2.2. RNA extraction procedures

Virions were concentrated from infected tissue culture fluid with 7% polyethylene glycol 8000 in the presence of 2.3% NaCl, and centrifuged through a 30% sucrose cushion. RNA was isolated from the virions by phenol/chloroform extraction followed by ethanol precipitation.

Extraction of total cytoplasmic RNA was performed according to Gough (1988). Virus-infected or uninfected cultured cells were harvested and resuspended in 10 mM

Tris-HCl pH 7.5, 0.15 M NaCl, 1.5 mM $MgCl_2$ and 0.65% NP-40. After centrifugation, an equal volume of 7 M urea, 1% SDS, 0.35 M NaCl, 10 mM EDTA and 10 mM Tris-HCl pH 7.5 was added to the supernatant. This was followed by phenol/chloroform extraction and ethanol precipitation.

Two methods for preparing templates from pooled human serum samples (negative for dengue antibodies by HI and IgM ELISA) spiked with a known quantity of dengue virus were compared. In the modified method of Chungue et al. (1993), 4 μl of acid-washed, size-fractionated silica and 100 μl of buffer containing 4 M guanidine isothiocyanate, 40 mM Tris-HCl pH 6.4, 17 mM EDTA pH 8.0 and 1% Triton X-100 were added to 10 μl of spiked human serum. The suspension was vortexed briefly, allowed to stand at room temperature for 10 min, vortexed again and pelleted by centrifugation. The silica pellet was washed twice in 100 μl of washing buffer (50% ethanol, 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 50 mM NaCl) and finally in 100 μl of water. The pellet was resuspended in 20 μl of water containing 25 U of RNase inhibitor and incubated at 56°C for 10 min and the supernatant added to 30 μl of RT-PCR mix. The other method used was modified from Morita et al. (1991). 5 μl of spiked human serum was mixed with 5 μl of detergent mix (containing 1% NP-40, 50 U RNase inhibitor), incubated at room temperature for 1 min and added to 40 μl of RT-PCR mix.

2.3. Clinical serum samples

A retrospective study was carried out on acute sera from 13 dengue patients confirmed by virus isolation. The sera had been stored frozen at −20°C for 2–6 months. Convalescent sera were available for four patients and they were classified as one primary and three secondary infections by HI tests. RNAs were extracted by the modified method of Chungue et al. (1993) and subjected to RT-PCR. Concurrently, virus reisolation was attempted from the sera by inoculation into C6/36 cells followed by immunofluorescent staining with monoclonal antibodies.

2.4. Oligonucleotide primers

Nucleotide sequences of the NS3 region flanked by consensus primers DV1 and DV3 (Chow et al., 1993) of dengue virus type 1 (Fu et al., 1992), type 2 (Deubel et al., 1988; Hahn et al., 1988; Irie et al., 1989; Blok et al., 1992), type 3 (Osatomi and Sumiyoshi, 1990) and type 4 (Mackow et al., 1987) and Japanese encephalitis virus (Sumiyoshi et al., 1987; Aihara et al., 1991) were aligned and compared using the DNASIS (eighth version) software programme (Hitachi, USA). Regions demonstrating maximal intra-serotype homology and minimal homology against the genomes of the other dengue virus types and other flaviviruses were searched. Four type-specific downstream primers with similar melting temperatures were designed, generating a characteristically sized product for each dengue virus type after amplification with the consensus upstream primer DV1 (Table 1). These oligonucleotides were synthesized chemically and purified

Table 1
Nucleotide sequences and positions of upstream consensus (DV1) and type-specific downstream (DSP1-4) primers

Primer (and orientation)	Sequence	Nucleotide position	Target band sizes (bp)
DV1 (+)	5'-GGRACKTCAGGWTCTCC-3'		
DSP1 (−)	5'-AGTTTCTTTTCCTAAACACCTCG-3'	5067-5045	169
DSP2 (−)	5'-CCGGTGTGCTCRGCYCTGAT-3'	5279-5260	362
DSP3 (−)	5'-TTAGAGTYCTTAAGCGTCTCTTG-3'	5174-5152	265
DSP4 (−)	5'-CCTGGTTGATGACAAAAGTCTTG-3'	5342-5320	426

K, G/T; R, A/G; W, A/T; Y, C/T

2.5. RT-PCR conditions

A conventional two-step RT-PCR was performed in which RT using the four downstream primers was followed by inactivation of reverse transcriptase and by PCR incorporating the upstream consensus primer DV1.

Various RT-PCR parameters were examined individually for a simplified RT-PCR protocol combining RT and PCR in the same tube. 1 ng of purified dengue viral RNA was added in a final volume of 50 μl containing 1 × PCR buffer with 1.5–3.0 mM $MgCl_2$, 0.15 μM each of the four downstream primers and the upstream primer, 0.2 mM of each dNTP, 10 U RNase inhibitor and 25 U MMLV reverse transcriptase and 0.1–0.5 U of Super *Taq* polymerase (HT Biotechnology, UK). RT was carried out at 42, 46, 50, 55 or 60°C for 15 min. This was followed by an initial denaturation at 95°C for 1 min, and 30 or 35 PCR cycles of denaturation at 95°C for 0.5 min, annealing at 50, 55, 60 or 65°C for 0.5 or 1 min and extension at 72°C for 0.5 or 1 min, with or without a fixed ramp time of 0.5 or 1 min between each segment. This combined RT-PCR protocol was also tested using the "hot-start" technique whereby the RT-PCR mix (except the five primers) was pre-heated to 55°C for 2 min. The five primers which were pre-added in the tube lid were then added to the mix by a quick centrifugation.

2.6. Evaluation of RT-PCR specificity and sensitivity

The specificity of the assay was determined using the optimized RT-PCR protocol for 0.1 or 1 μg of total cellular RNA of uninfected and dengue, Japanese encephalitis, Kunjin and yellow fever virus-infected C6/36 cells.

The sensitivity of the RT-PCR assay for each dengue virus type was determined using plaque-titrated dengue virus type 1, 2 and 4 viruses, while dengue type 3 virus was titrated by median tissue culture infective dose ($TCID_{50}$). Plaque titration was performed in Vero cells in M199 growth medium supplemented with 5% FCS, and overlaid with carboxymethylcellulose overlay medium (Gould and Clegg, 1985; Morens et al., 1985). $TCID_{50}$ titration of dengue 3 virus-infected C6/36 cells was performed as described by Minor (1985) with modifications, and the $TCID_{50}$ endpoint calculated according to Karber (1931). 10 μl of 10-fold serial dilutions of virus in tissue culture fluid were mixed with an equal volume of 1% NP-40 containing 25 U of RNase inhibitor and

incubated at room temperature for 1 min (Morita et al., 1991). RT-PCR was then performed in a 50 μl volume under optimized conditions.

2.7. Analysis of PCR products

10 or 20 μl of each amplified product were electrophoresed on a 2% agarose gel in 1 × TBE buffer containing 0.5 μg/ml ethidium bromide. The amplified bands were directly sequenced using the corresponding specific downstream primer as sequencing primer as previously described (Chow et al., 1993).

3. Results and discussion

3.1. RT-PCR analysis

The RT-PCR conditions were initially studied using 1 ng of purified viral RNA of each dengue serotype. It was found that the optimized RT-PCR conditions were 1 × PCR buffer with 1.5 mM $MgCl_2$, 0.15 μM of each primer, 0.2 mM of each dNTP, 25 U of reverse transcriptase and 0.5 U of *Taq* polymerase subjected to RT at 50°C for 15 min followed by an initial denaturation of 95°C for 1 min, 10 PCR cycles of 95°C for 0.5 min, 50°C for 1 min and 72°C for 1 min with a ramp time of 1 min, and 20 or 25 PCR cycles of 95°C for 0.5 min, 50°C for 0.5 min and 72°C for 0.5 min with a ramp time of 0.5 min. Amplification using the one-step RT-PCR with "hot-start" was found to be of comparable sensitivity and improved specificity compared to the conventional two-step RT-PCR protocol. Furthermore, this single-step RT-PCR protocol obviates the need to open the tube to add PCR mix to the RT mix, thereby avoiding potential cross-contamination of samples.

Target bands of expected sizes were amplified by RT-PCR of each of the four dengue virus types (Fig. 1, lanes 1–4). No target bands were obtained for Japanese encephalitis, Kunjin, yellow fever viruses and uninfected C6/36 cells (Fig. 1, lanes 5–8) indicating the specificity of this assay for dengue viruses and the absence of cross-reactivity with related flaviviruses.

DNA sequences of PCR products of dengue viruses obtained by direct sequencing using the corresponding specific downstream primers concurred with the published sequences, thereby confirming the authenticity of each of the target bands.

The sensitivity of the RT-PCR assay was 3.0 plaque-forming units (PFU), 1.4 PFU, 0.1 $TCID_{50}$ and 3.5 PFU for dengue virus types 1, 2, 3 and 4, respectively (Fig. 2, lanes 1–12). $TCID_{50}$-titrated dengue 3 virus was used in this study as this strain did not yield high plaque titres.

3.2. Application to virus-spiked and actual clinical serum samples

Using human serum spiked with dengue 2 virus, two methods of template preparation for RT-PCR were assessed. The method described by Morita et al. (1991) which

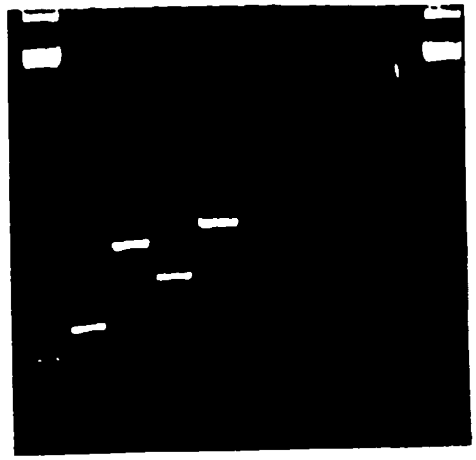

Fig. 1. Specific typing of dengue viruses by single-step RT-PCR of cytoplasmic RNAs of virus-infected and uninfected C6/36 cells. Agarose gel electrophoresis of RT-PCR products demonstrating diagnostic target sizes of 169 bp, 362 bp, 265 bp and 426 bp for dengue virus types 1, 2, 3 and 4, respectively (lanes 1–4), which are absent for Japanese encephalitis, Kunjin, yellow fever viruses and non-infected C6/36 cells (lanes 5–8), illustrating good specificity. M is a 123 bp ladder size marker.

sensitivity, i.e. 1.4×10^3 PFU was undetectable (data not shown). However, the method of Chungue et al. (1993) achieved a far higher sensitivity of 14.2 PFU of dengue 2 virus (Fig. 2, lanes 13–15). Although the method of Morita et al. involves minimal handling

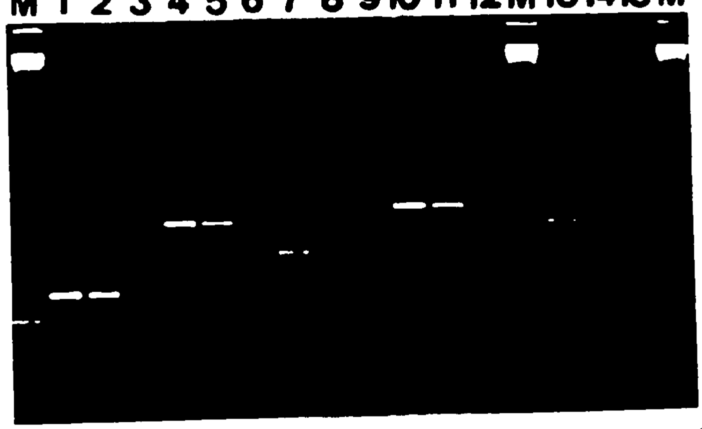

Fig. 2. Sensitivity of RT-PCR typing of dengue viruses. Gel electrophoresis of RT-PCR products of about 100, 10, 1 PFU of dengue virus type 1 (lanes 1–3), type 2 (lanes 4–6) and type 4 (lanes 10–12), of 10, 1, 0.1 $TCID_{50}$ of type 3 (lanes 7–9); and RT-PCR products of RNA from human serum spiked with 1000, 100, 10 PFU of dengue virus type 2 (lanes 13–15). M is a 123 bp ladder marker.

of the serum and saves time and cost, its low sensitivity may be due to the presence of inhibitors of both reverse transcriptase and *Taq* polymerase in the serum. While the method of Chungue et al. requires more manipulations, it has a higher sensitivity probably due to the removal of non-specific inhibitors in the serum. The latter is thus a method of choice particularly in cases of low viraemia where high sensitivity is required.

Re-isolation of dengue virus from the 13 serum samples was unsuccessful. This could be due to inactivation of the virus resulting from prolonged storage of the sera for several months at −20°C and their repeated freeze-thawing before the study. Notwithstanding this, five out of the 13 sera were positive by RT-PCR. These five sera included two out of the three sera from patients with secondary infections and one serum sample from a patient with a primary infection. Of the five positive sera, four were identified as dengue 2 virus and one as dengue 3 virus, in complete concordance with the initial virus isolation and immunofluorescence data. Compared with virus re-isolation, RT-PCR was therefore exquisitely more sensitive and could even detect non-viable viruses present in the more viraemic serum specimens.

Dengue virus infections are becoming an increasingly important international health problem with explosive outbreaks occurring in many parts of the world. A rapid, sensitive and specific tool for both diagnostic and epidemiological purposes is therefore needed. Although other researchers have reported PCR detection of dengue viruses using type-specific primers, our single-step, five-primer RT-PCR-based assay for the simultaneous detection and typing of dengue virus infections is rapid, requires fewer manipulations and yet exhibits similar high sensitivity and specificity. Work is underway to apply this RT-PCR assay for studying more serum samples and as well as mosquito vectors, in parallel with established routine laboratory techniques.

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Identification of mosquito-borne flavivirus sequences using universal primers and reverse transcription/polymerase chain reaction

V. Pierre, M.-T. Drouet and V. Deubel (*)

Unité des Arbovirus et virus des fièvres hémorragiques, Institut Pasteur, 75724 Paris Cedex 15

SUMMARY

A reverse transcription/polymerase chain reaction (RT/PCR) protocol for the rapid detection and identification of flaviviruses was developed using a set of universal oligonucleotide primers. These primers correspond to sequences in the 3' non-coding region and in the NS5 gene which are highly conserved among the mosquito-borne flaviviruses. The sequences of the resulting amplified products were analysed for dengue 1, dengue 2, dengue 3, dengue 4, Japanese encephalitis, West Nile, yellow fever and Zika viruses, and compared with the published sequences of other flaviviruses. The 291-297 nucleotides corresponding to the C-terminus of NS5 gene showed 56 to 76 % similarity, whereas the 3' non-coding region (190 to 421 nucleotides) showed only 20 to 36 % similarity. Genetic classification of the Zika virus supported its traditional serological grouping. Recombinant plasmids containing the flavivirus sequences were used in a nucleic acid hybridization test to identify the RT/PCR products derived from viral RNA extracted from experimentally infected mosquitoes. The plasmids were dotted on a strip of nitrocellulose membrane and incubated with the RT/PCR product labelled with digoxigenin during the PCR step. This is a valuable method for the rapid and specific identification of mosquito-borne flaviviruses in biological specimens and for subsequent sequence analysis.

Key-words: Flavivirus, Sequencing, PCR, RT; Virus identification, Epidemiology.

INTRODUCTION

Flaviviruses are a group of at least 68 viruses classified into eight serosubgroups and nine individual serotypes which replicate in vertebrate hosts (Calisher *et al.*, 1989). About two thirds of them are transmitted by haemophagous mos[illegible]

to be one of the major reservoirs for the viruses, which can be maintained in nature via enzootic circulation (Monath, 1990). Fifteen mosquito-borne flaviviruses are human pathogens and are usually associated with non-differentiated afflictions, including fever, headache and arthralgia. Some viruses, like yellow fever (YF), dengue [illegible]

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(*) Corresponding author.

(WN) and Zika (ZIKA) viruses give specific pathogenic patterns, including encephalitis, hepatitis and haemorrhages, and cause major public health problems in tropical areas where epidemics occur (Monath, 1990). Early identification of the aetiological agent of these epidemics would help the rapid instigation of adequate control strategies for the high-risk non-immune population. Flaviviruses are usually recovered from entomological or biological samples by intracerebral inoculation of suckling mice and by infection of susceptible mosquito and monkey cell lines (Kuno *et al.*, 1985). Flaviviruses generally do not have a direct pathogenic effect on vectors, and the virus can be isolated seven days after the infectious blood meal. However, human viraemia lasts for 3-5 days and is usually neutralized by specific antibodies by the time specific symptoms appear. DEN viruses can be detected in the presence of neutralizing antibodies by amplification of the viral genome by reverse transcri ion (RT) coupled to polymerase chain rea on (PCR) (Deubel *et al.*, 1990; Henchal *et al* 1991). Moreover, RT/PCR diagnosis of flaviv s considerably quicker and more sensiti than the usual tests for virus identification (Deubel *et al.*, 1990; Eldadah *et al.*, 1 : Henchal *et al.*, 1991; Howe *et al.*, 1992; ita *et al.*, 1991; Lanciotti *et al.*, 1992; Porter *al.* 93; Tanaka, 1993; Tardieux and Poupe 199 Trent and Chang, 1992).

The single-stran . positive-sense flavivirus genome is about 11. cleotides long and has no 3'-poly(A) tract. ontains at its 5' and 3' extremities two n oding regions of about 100 and 500 nucleotides, respectively. The genome comprises a single long open reading frame encoding a polyprotein: 5'-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3', which is cleaved co- and post-translationally (Chambers *et al.*, 1990). Nucleotide sequence similarities among flaviviruses of different subgroups can be lower than 40 %. Sequences of virus genotypes of a single virus show more than 20 % intratypic variation (Rico-Hesse, 1990). It is therefore difficult to find sequences conserved among different flaviviruses within the genus for selection of oligonucleotide primers for RT/PCR. This limits the number of flaviviruses that can be tested at the same time. For DEN diagnosis, Henchal *et al.* (1991) and Lanciotti *et al.* (1992) established a single consensus set of oligonucleotide primers to replace the four sets of primers previously used (Deubel *et al.*, 1990; Morita *et al.*, 1991).

In this study, we have used a single set of primers for universal amplification of flaviviruses highly pathogenic for human. They are based on conserved elements in the 3' untranslated region of mosquito-borne flavivirus RNA (Hahn *et al.*, 1987; Wengler and Castle, 1986) and in the protein NS5. The product amplified by PCR can be identified by using it to probe a set of recombinant plasmids each containing the corresponding sequence from a different virus. Using this technique of one-round RT/PCR and hybridization, we characterized genomic RNA from YF, DEN, JE, WN and ZIKA viruses in mosquito samples within two days. The sequences amplified from these viruses were also analysed.

AP61 = *Aedes pseudoscutellaris* clone 61.
cDNA = copy deoxyribonucleic acid.
CS2 = consensus sequence 2.
DEN = dengue.
dig = digoxigenin.
JE = Japanese encephalitis.
m.o.i. = multiplicity of infection.
PCR = polymerase chain reaction.
RT = reverse transcriptase, reverse transcription.
rpm = rotation per minute.
SDS = sodium dodecyl sulphate.
SSC = sodium salt citrate.
Taq = *Thermus aquaticus*.
TNE = Tris-NaCl-EDTA buffer.
WN = West Nile.

MATERIALS AND METHODS

Viruses

Viral strains used in this study (DEN1: reference Hawaii 1944; DEN2: reference New Guinea C NGC 1944; DEN3: Thai PaH881/1988; DEN4: reference Caribbean H241/1971; YF: Senegalese French neurotropic virus FNV/1927; WN: reference Egyptian E101/1962; JE: reference Nakayama/1935; ZIKA: Central African Republic ArB 7630/1976) were obtained from brain tissues and propagated in *Aedes pseudoscutellaris* (AP61) cell monolayers. Viral suspensions in AP61-infected supernatant were titrated by plaquing on porcine PS cells (De Madrid and Porterfield, 1969).

Virus growth and RNA extraction

Viruses were grown in AP61 cells (m.o.i. = 1-5 PFU/cell) for three days in Leibovitz L15 medium supplemented with 10 % foetal calf serum, 10 % tryptose phosphate and antibiotics. Cells were washed in 1 × TNE (0.5 M Tris-HCl pH 7.2, 0.13 M NaCl, 1 mM EDTA) buffer and lysed at 4°C in 0.1 × TNE containing 0.5 % NP40. Cell nuclei were pelleted by centrifugation at 2,500 rpm and the cytoplasmic extracts were deproteinized by three phenol treatments in the presence of 1 % SDS. RNA were precipitated with 2.5 volumes of pure ethanol/0.3 M ammonium acetate.

Male *Aedes aegypti* mosquitoes were infected by intrathoracic inoculation with virus-infected AP61 supernatants (Rosen and Gubler, 1974). After 10 days of incubation, 10 mosquitoes were ground in 1 ml of Hank's medium containing 0.75 % bovine serum albumin. RNA was extracted using a modification of a method previously described by Chomczinski and Sacchi (1987). Briefly, after centrifugation at 3,000 rpm at 4°C, a 200-µl aliquot of supernatant was mixed with an equal volume of lysis buffer (8 M guanidine isothiocyanate, 50 mM Na citrate pH 7.0, 1 % sarkosyl, 0.1 M β-mercaptoethanol). The mixture was phenol-extracted by adding 40 µl of 2 M Na acetate pH 4.0, and 615 µl phenol:chloroform mixture (7:3 w/v). After centrifugation for 15 min at 4°C, the aqueous phase was collected and RNA precipitated with an equal volume of ice-cold isopropyl alcohol. The RNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4°C, washed with 75 % ice-cold ethanol, and vacuum-dried. RNA was resuspended in 40 µl of sterile distilled water just pri- ... DNA priming.

CS2 highly conserved among several flaviviruses and located about 100 nucleotides from the 3'-end (Hahn et al., 1987; Wengler and Castle, 1986; Deubel et al., 1988). The second oligonucleotide primer EMF1 encodes an amino acid sequence (WMTTEDM) conserved in all flaviviruses. Alternative bases .. the third position in some of the codons (in parentheses) were incorporated into the primer sequence ... CGGAT GAC(C,G)AC(G,T)GA(A,G)GA(C,T)ATG3' to enable hybridization to all known variat... s in this nucleotide in flaviviruses (Rice et al., ... ; Castle et al., 1986; Deubel et al., 1988; F... , 1992; Mackow et al., 1987; Osatomi and S... ni, 1990; Sumiyoshi et al., 1987).

RT/PCR was performed ... 5 µg of AP61-infected cell RNA or 10 µl of ... spension (mosquito and ~~serum samples)~~ ... was amplified by RT/PCR following the met... previously described (Deubel et al., 1993). Brie... RNA was mixed with 1 µl (100 ng) of VD8 prir... d heated at 90°C for 2 min, and placed or ... was synthesized in 20 µl of 50 mM Tris-H... pH ... , 50 mM KCl, 8 mM $MgCl_2$, 10 mM dithi...rei... 0.2 mM of each dATP, dCTP, dGTP and dT... 40 units "RNasin" (human placental ribonuclea... nhibitor, Promega) and 2 units of reverse trans... ase (RT) from avian myeloblastosis virus (B... hringer-Mannheim). The reaction mixture w... ated for 1 h at 42°C.

cDNA was amplified by PCR by ma... g 4 µl of the cDNA sample up to a 50-µl final ... ume of 50 mM KCl, 10 mM Tris-HCl pH 8. , 2 mM $MgCl_2$, 10 µg gelatin, 0.2 M of each of the four deoxynucleotides, 300 ng of each of the primers and 0.5 units of *Thermus aquaticus (Taq)* polymerase (Perkin Elmer Cetus). After 5 min of denaturation at 95°C, the mixture was subjected to 30 PCR cycles: 95°C for 30 s, 53°C for 90 s and 72°C for 60 s; followed by a final 10-min polymerization step at 72°C. For direct detection of viral material in the samples, the cDNA was labelled during amplification by adding to the PCR mixture 0.025 mM of dUTP-11-digoxigenin (dig) (Boehringer-Mannheim) and 0.175 mM dTTP instead of 0.2 mM of dTTP. DNA products were purified by precipitation in 3 volumes of ethanol containing 0.125 M LiCl and 6.2 mM EDTA at −80°C for 30 min. DNA were pelleted by centrifugation at 10,000 rpm for 10 min at 4°C, rinsed in 75 % ice-cold ethanol, vacuum-dried and resuspended in 50 µl of distilled water.

Cloning flavivirus cDNA

The sequence of primer ... (TAACCTCTAG3' is complementary to a sequence

... precipitated in 2.5 volumes of pure ethanol ...

NaCl. DNA were pelleted by centrifugation at 10,000 rpm for 10 min at 4°C, rinsed and dried. The extremities were phosphorylated with polynucleotide kinase (Boehringer-Mannheim) following the procedure described by Maniatis *et al.* (1982). The DNA were cloned into *Eco*RV- and *Hinc*II-cut, calf intestine phosphatase-dephosphorylated plasmid bluescript pKS(+) (Stratagene (Maniatis *et al.*, 1982)). The polylinker of this plasmid is framed by phage T3 and T7 promotor sequences. Double-stranded recombinant DNA was sequenced by the dideoxynucleotide method using T3 and T7 universal primers and either T7 polymerase (Sequanase, US Biochemicals) or *Taq* polymerase (Promega) sequencing kits as previously described (Deubel *et al.*, 1993).

Dot-blot hybridization

DNA products obtained from the samples were labelled during amplification and used to probe for hybridization with various recombinant plasmids. Plasmids each containing sequences from a different flavivirus were linearized with *Bam*HI, denatured and transferred to nitrocellulose membranes as previously described (Deubel *et al.*, 1990). The membranes were prehybridized in 4×SSC, 5 % non-fat milk, 50 % formamide and 0.1 % SDS as previously described (Deubel *et al.*, 1990) and hybridized overnight at 42°C in the same solution containing the labelled DNA product. Membranes were washed 4 times for 10 min in 2×SSC containing 0.1 % SDS at 20°C and twice in 0.2×SSC and 0.1 % SDS at 72°C for 40 min, and rinsed in 1×TNE. Membranes were then incubated for 30 min in TNE containing a 1/5,000 dilution of alkaline-phosphatase-labelled anti-dig antibodies (Boehringer-Mannheim) and washed in TNE. The enzyme was revealed as previously described (Deubel *et al.*, 1990) to visualize positive hybridization.

Dendrogram and sequence similarity

A phylogenetic tree generated by multiple nucleotide sequence alignments was calculated using the "CLUSTAL V" program (provided by D. Higgins, Heidelberg, Germany) (Higgins and Sharp, 1988). The dendrogram was calculated in two stages: (1) a similarity score was recorded by comparing all pairs of sequences using the fast/approximate method of Wilbur and Lipman (1983); (2) these ... the dendrogram using

RESULTS

Selection of oligonucleotide primers and evaluation of their specificity

Comparative analysis of the nucleotide sequences of mosquito-borne flaviviruses identified two sequences CS2 and EMF1, each sharing more than 90 % identity among YF, DEN, JE and WN viruses (Rice *et al.*, 1985; Castle *et al.*, 1986; Deubel *et al.*, 1988; Fu *et al.*, 1992; Mackow *et al.*, 1987; Osatomi and Sumiyoshi, 1990; Sumiyoshi *et al.*, 1987). One of these sequences identified at the 3'-end of the flaviviruses, CS2, is 20 nucleotides long (Hahn *et al.*, 1987). It is repeated identically approximately 70 nucleotides upstream in viruses of the DEN and JE serogroups, but is present only as a single copy in YF virus. Tick-borne viruses do not contain this sequence (Mandl *et al.*, 1989; Pletnev *et al.*, 1990). Primer EMF1 matched a flavivirus-specific sequence located 500-700 nucleotides upstream from CS2 and encoded a series of amino acids corresponding to codons of low or no degeneracy (see "Materials and Methods"). The sequence of ZIKA virus was not available and we did not know if the sequences corresponding to the primers were present in its genome. To test the specificity of the two oligonucleotide primers VD8 complementary to CS2, and EMF1, RNA isolated from DEN1-, DEN2-, DEN3-, DEN4-, ZIKA-, YF-, WN- and JE-infected AP61 cells were assayed by RT/PCR. The amplified products were visualized by staining with ethidium bromide after agarose gel electrophoresis (fig. 1). RNA from the eight flavivirus-infected cells were amplified, including that of ZIKA virus. The sizes of the amplified DNA (500-700 base pairs) were consistent with those predicted from the published sequences. A doublet band was obtained for DEN, JE and WN viruses.

The size of the amplified fragments was variable (fig. 1). The length of NS5 is highly conserved within the flavivirus genus (Tolou *et al.*, 1992), suggesting that the variability was due to heterogeneity in the position of VD8 or in the

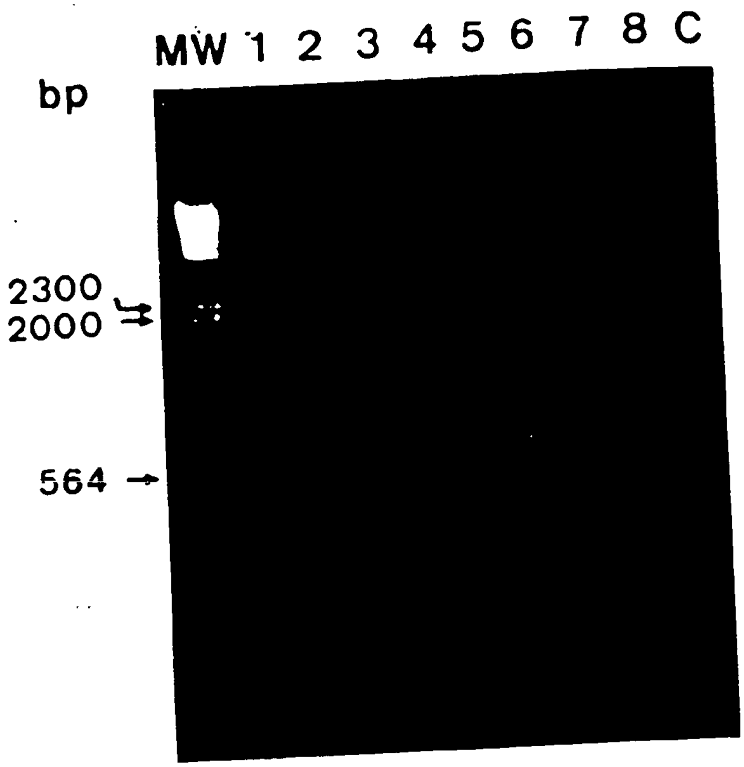

Fig. 1. Amplification products.

RNA from mosquitoes infected with DEN1 (lane 1), DEN2 (lane 2), DEN3 (lane 3), DEN4 (lane 4), ZIKA (lane 5), YF (lane 6), WN (lane 7), and JE (lane 8), or from mock-infected mosquitoes (C) was amplified by PCR using VD8 and EMF1 primers (see "Materials and Methods"). Agarose (1 %) gel stained with ethidium bromide.

with RNA from cells infected with tick-borne Langat virus, Bunyamwera bunyavirus or Chikungunya togavirus (data not shown).

Sequencing the products amplified from the VD8 and EMF1 primers

The amplified DNA products were purified on low melting point agarose gel, cloned into Bluescript pKS plasmids and sequenced using T3 and T7 universal primers. The alignment by the ... Higgins and Sharp ...

... the NS5 sequence ... WN virus gene. The 3' non-coding region was more divergent in both length and sequence than the NS5 gene (table I). The ZIKA virus shows 56 to 60 % similarity in the NS5 gene region and 20 to 31 % similarity in the 3' non-coding region compared to the other viruses. This is consistent with the serological classification which distinguishes ZIKA virus from the other mosquito-borne flaviviruses (Calisher *et al.*, 1989). Figure 3 shows the phylogenetic tree of the flaviviruses deduced from the amplified sequences. Representative viruses within the DEN and JE serogroups were 59 to 70 % identical, whereas only 40 to 45 % similarity was observed between serogroups. The highest divergence wi... ... observed for two WN ... other viruses ...

Specificity and sensitivity of the probes for hybridization of amplified viral cDNA

The extended homology in the NS5 coding region of the DEN serocomplex (table I) could lead to cross-reactivity between the strains when these sequences were used for specific hybridization. Therefore, the cloned DEN sequences in the recombinant plasmids were partly deleted to remove highly conserved fragments in NS5, using appropriate restriction sites (see "Materials and Methods" and fig. 2). The plasmids containing the cloned sequences for JE, WN, ZIKA and YF were used undeleted for strain identification. The specificity of these sequences was confirmed by hybridization on amplified cDNA obtained from infected mosquito RNA extracts (data not shown).

The use of numerous plasmids each containing a flavivirus-specific sequence to probe an RT/PCR sample for identification would be laborious and time-consuming. Moreover, the use of radiolabelled probes is unsuitable for routine diagnosis. Therefore, RT has been carried out by dotting each of the recombinant plasmids on a nitrocellulose strip (fig. 4). The optimal concentration of the plasmid was determined to be 100 ng per dot (see below). The "ready to use" strip was incubated with the amplified DNA labelled with dig by including dUTP-11-dig during the amplification step. The amplicons could therefore be tested by hybridization against the eight plasmids. Each RNA extract obtained from flavivirus-infected mosquitoes was subjected to RT/PCR using dUTP-dig and hybridized with a "ready to use" strip. The DNA-dig retained on nitrocellulose by hybridization with one of the cloned sequences was detected with alkaline-phosphatase-labelled anti-dig antibody. Figure 4 shows the reactivity of each amplified flavivirus cDNA with a "ready to use" strip, and the specificity of the target sequence for each of the eight viruses tested.

The sensitivity of the method was established using the DEN2 virus. To do this, RNA from serial dilutions of plaque-titrated viral particles was extracted for RT/PCR. The amplified DNA-dig products were hybridized with nitro-

Table I. Percentage identity between flavivirus in NS5 gene fragment and in 3' non-coding region.

	DEN1	DEN2	DEN3	DEN4	YF	ZIKA	JE	WN
DEN1	—	72 (*)	74	67	60	60	64	69
DEN2	30 (**)	—	76	70	61	59	63	66
DEN3	30	35	—	69	58	57	62	62
DEN4	30	36	35	—	60	59	61	60
YF	30	23	28	27	—	56	56	57
ZIKA	31	25	20	2[illegible]	26	—	57	59
JE	22	29	26	25	27	28	—	71
WN	24	28	27	27	29	23	25	—

(*) The C-terminus of the NS5 gene only; (**) the 3' non-coding region only.

Fig. 2. Aligned nucleotide sequences of flavivirus-amplified products.

cDNA fragments of the NS5 C-terminus and 3' non-coding fragment from eight flaviviruses were cloned in pKS Bluescript and sequenced (see "Materials and Methods"). The first and last nucleotide in each sequence are indicated. Overlapping sequences with the VD8 and EMF1 primers are underlined. Residues identical to the sequence of DEN1 virus are shown as dots. Conserved nucleo- [illegible] serogroups are overlined. In-frame termina- [illegible]

```
             TGGATCACGGACGGAAGACATGTTGTCACTGTGGAATAGGGTTTGGATACAGGA.AACCCATGGATGGAGGATAAAACCCATGTATCCAGTTGGGAAGATG
DEN1  (1)    ......G.........A.  .C.   .C...  G.    TC A     T      ..A..C.    T.CA. GGAATCA     C  AA
DEN2  (1)    ........G.......C.TA.T  C..   C.....G....      C  T.          .A. C..  .T CA..CA A C
DEN3  (1)    .....C ....G.....C.CAA        C..A. G          A  C     TAAT  ACT .C. G  T.CA  TCATTCG          CA
DEN4  (1)    ... C .T... .T . C TGAG       C. .A. A      .ACCA C      CAC   C     C. G  AATG. GAAAGAA    AG
YF    (1)    .........G.      C CATG      ...A..G      T      G   GA CAT         C  G  T.C..  A.A AG    AC    CA
ZIKA  (1)    .....T. G..     .C CAG       C..A..CG.T  T  A       TGA        .AT . C. G  T CAA.TA A      AC.
JE    (1)    ..C.........      C. GAG  C  CC.T.....          G. TGA         .A. C.      CA. CGAG AA    AGT  C
WN    (1)    ..............
               EMF1 ->

             TTCCATACTTAGGAAAAAGGGAAGATCAGTGGTGTGGATCCCTGATAGGCTTAACAGCAAGGGCCACCTGGGCCACTAATATACAAGTGGCCATAAACCA
DEN1         .C........G. ......A.....C..A.....C..C..A.....C..GC.G...AGC...  T........G.AG..C..C. GACA..A.....T.
DEN2         .......C...  G..G..A.........A.......C.....A..C..C..TC.C..TT.C. A..A.........CAG..C....TCACA. A. CC A..
DEN3         .A..T...C..........A......TT...............T....T..AC.TT.TT.C..............G.AG..C..T. CACA.    ..C ..
DEN4         .C..T..TC..ACC..G..AC.....CA..CT...C.......A.....T..AA.G..CAAT...............T.CC.C..C..TT.. T.. CC.T.G
YF           .C..C..TC.G.............CTTA...............T....GCAC.GGC.CC.AC....T.....TGAG..C..CA....ACA.AG.C...AT
ZIKA         ....G...G.G.....GC.T. G..CATC.........CAG...T..T..AACGCG.T.C  A..A.......TGAG. C..CT.T C.  G. ..
JE           .C.......C.........C....G..CATC........CAG.T....T.. AC. G.A.CC.A...  ..G.....AGAA..C..C..G..A    .C ..
WN           

             AGTGAGAAGGCTTATTGGGAATGAGAATTATCTA---GATTACATGACATCAATGAAGAGATTCAAGAATGAGAGTGATCTCGAAGGGGCACTCTGG---TAA
DEN1         ...C...TCC..C......C..     AG.A...AC.---..........C...  C  ....A.......GA.GA..AGAG..AGAG.C....A.TTT G.....---..G
DEN2         G.........C.....A. CG.....AG.G.T...G---..C.......C.T..G..   ..        .G...G...GAG..GTCA..G  A   CA.T......---...
DEN3         G..C..G.AC..G..C.....A...G.G...G.G---..........C..GTC.....A.....A..GCGC.CTTTTC..GAGT.....A.TT  .G------...
DEN4         TA.CC...C...GG....AC.G.....A....ACT---...C...C.A....GTC...G.C....ATTCTGTG..TGC....C..GC...CC..CTGAGCTTATC.G.
YF           G...C.C...A.C..A..TG.....AG.AA.ATACATG..C....T.AT.CA.CCA.GTCC...G.TT.GG.....GAA.GGTC.AC.CCT.G.G.G.T.---...
ZIKA         G..T....GCT..C.........A..A......G.T---...C.......A..T...C.C.G.....ATG.AG.C.TCTTGATC.AG.....ACAGGG..ATC---..G
JE           ...C...GCAA.C.....AG........G...G.G---..............GT....T.A....  ..ATG.AG.CAC..C.TTAG.TAG..ACA..G.ACT.---...
WN           

             -G------TCA------------------AC----ACATTCACAAAACAA-AGGAAAATAAGAA-ATCAAACAAGG------CAAGAAGTCA-G--GCC--
DEN1         -------------------------------------------...G.------------------.AA......A.------.C.A.....  - --.T.--
DEN2         -----------------------------------------------...G-....  .G-...A..-.--G.G--...-------...ACT.  . -.--. --
DEN3         ------------------------------------------------------------------------------------------------------
DEN4         --------.T.------------------C.----.------.....-------------------..........----------------------------
YF           AATACCATCT.ATAGGAATAACCGGGATA.A-AAC..CGGCTGG.GA.CC...ACTCCCC.C..CT.G.....CG..ATATAAACCACG.CTGGAGAA...--
ZIKA         CA------C..------------------TTTTAGTG..GT..GCCTGC-TA.CC.GCC.C.G-T.TGGGA..C.------TGT.C..C.T-.TA...--
JE           T.--TGAT.T.AAGTAGGAAAATAAACC.TGTAA...-ATGT...-------------.G...---------.T.T--GTG..TATG.------A.T.--
WN           -A--TACT.T.TTAATTGTAAATAGACA.TGTAAG...GTGT...GT.T-..TTTT...GT.GC..TT.GTG.T.TTAGTGT..AT...T.A.AAATTTA

             ------GGATTAAGCCATAG------TACGGTAAG-------AGCTATGCTGCCTGTG----AGCCCCG---------------------TC---TAAGG
DEN1         ------.A...........------.....A..A-------.........A.....----.......-----------------------.---C....
DEN2         ------ACC.........C..------.....A.GA-------....G...A......----.....-----------------------. ---C....
DEN3         ---------------..A..------C.----..A-------.AT.------------------------------------------.T---.G...
DEN4         ----GGACTCCGCA.TTA.AAT--GAA..A.A..CCGGGATA.A-------AA..AC.GAT--------GGAGAACCGGACT-CCA-----.ACA.TGA.
YF           ------CC.GG.GA.GC.G.------A.ACCA.C.-------CAT.T....AG.C.A.----.A.G..A--------------------- GGCACG.A.
ZIKA         ------ATGCC.G--..A.AGCT-GCC..C.G.TACTGGGTAGA.GG.........C.TCTC..T...AGGAGGACTGGGTTAACAAATC.GACAAC..A
JE           AGGAGGAAG.C.G...GG.AGTTCGCC..C.G...TTGAGTAGA.GG......--..CAACTC.A....AGGAGGACTGGGTAACCAAAGC.G-CGA.GT.
WN           

             ACGT----AAAATGA-AGT-CAGGCC-GGAAG-------CCAC--------GGTTTGAG----------GAAACCGTGC-TGCCTGTA--GCTCCA----
DEN1         ....-TAA..G.A.TC..G-.CATTTTAA.T.--------...T--------A.C.........----------...TTG..-A......G--......----
DEN2         ....-TAA..G.A..-...-......C.A...--------............---------...-------...........-.........--.....G----
DEN3         CTA.-TGA.----.TC..G-.CACTT---G..--------...---------.C.......-----------............-..........--CG...G----
DEN4         ..A-GAAG..GT..TC..CC...AA.TCCACACGAG-TTTTG.-CACTGCTAAGC..T.AGGC-AGTG-.GG.T.G.ACA...GACCTCCAGGTTGCGA
YF           .A.C-CATGCCGCCTGT.A-GCCC.TC...G.-------A.........-------T.AG.T.A---------A....CCA.GC..T..G.--AGCG...--GG
ZIKA         .G..GAGA..GCCCTC..AA.C.T.TC....TAGGTCC.TG.TCACTGGAA...-..AAGACCAACGT.GG..ACA--AATT...GCCA...GCTGG
JE           .TCCATGT..GCCCTC..AA.C.T.TC....GAGGACC....ATGTTGTAAC. CA.AAGCCCAATGT.G...AC..TACGTC..GCCA.. TGC-GG
WN           

             ---------TC-GT----G-------GGGATGTAAAAA-CCCGGGA-------G--GCCT---G-----CAACCCATGGA--AGCTGT-ACCC-TGGTGG
DEN1         ---------C.T.A----.-------AA.G........AT.........-------G--...----A-----..A..........--......  . G.A. C.T
DEN2         ---------..-.----.-------.....C.....G--..T....-------G--.........-----.....A...........--.... -..G.AC....T
DEN3         ---------C.AA.AACG.-------.A.GC...T...TT...A..G-------A--.G.C---A-----TGCG...C....--.....  -..G G ..CAT
DEN4         AAAACCTGG.TTC.GG-GACCTCCCA----CCCC.G.GTAAAAA..ACGGA-----.  ..C-C.CTACC--....TCCC.CGT.G. GT.-----.AAA.
YF           A--------.GG.GAAAA.-------AA.G..GCG.CCTT..CCACCC---TTTAAT.TG----GGGCCTG.A.TGGA..CT.......-GAATC.CCA.T
ZIKA         GCAGTGCGGC.T.CG-CA.--CCCCA..AGGACTGGGTTA..AAAGCCG--TT.A-..  CC-CACGGCC...G..TC.TCTAG.A..CA.TAGAC.AG.T
JE           AGAGTGCAGT.T.CGATA.TGCCCCA..AGGACTGGGTTAA.AAAGGCAGATCAAC.  CCAC.CGGCC.T.G..C...TAAT.G..  T -A.CA..G.C
WN           

             -AAGC-A-TAGACTTAGAGGAGACCC (553)

JE           .T  A  G                  ...TTTAAAGT.A.A....A....A...AAA.......A.A.A.A.A.A.
WN           G   A  G.  VD8                                                             VD8
```

cellulose strips dotted with various concentrations of recombinant plasmid containing the cloned DEN2 sequence (table II): 50 PFU of DEN2 virus could be detected by hybridization with 10 ng of plasmid; 100 ng of plasmid could detect 5 PFU of virus. To compare the sensitivity of the method with cell culture, DEN2 virus from the same samples was identified after inoculation on AP61 cells. Cells inoculated with 5 PFU showed specific fluorescence in about 5 % of the cells seven days after inoculation (table II).

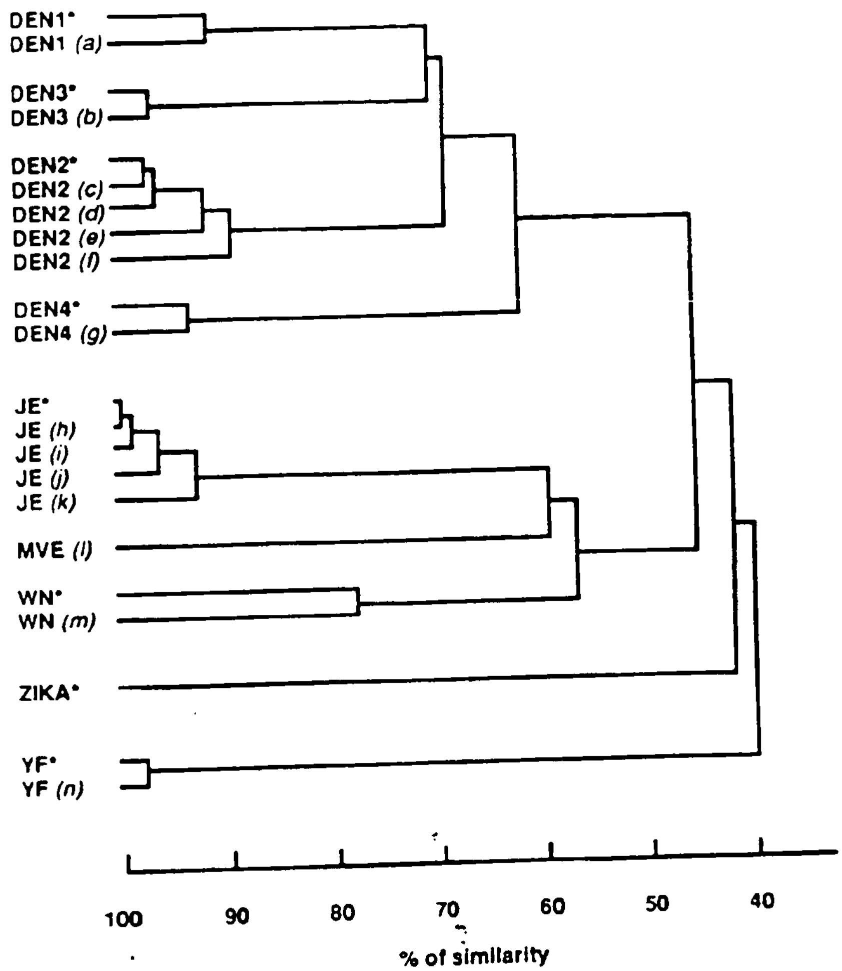

Fig. 3. Dendrogram presenting the extent of base sequence similarity between flaviviruses, in the sequence amplified by PCR using VD8 and EMF1 universal primers.

The sequences of different flavivirus genomes available from previous studies were included in the dendrogram for relationship analysis: (a) Fu *et al.* (1992), (b) Osatomi *et al.* (1988), (c) Block *et al.* (1992), (d) Irie *et al.* (1989), (e) Deubel *et al.* (1988), (f) Hahn *et al.* (1988), (g) Mackow *et al.* (1987), (h) Sumiyoshi *et al.* (1987), (i) Hashimoto *et al.* (1988), (j) Aihara *et al.* (1991), (k) Nitayaphan *et al.* (1990), (l) Lee *et al.* (1990), (m) Castle *et al.* (1986) and Wengler and Castle (1986), (n) Rice *et al.* (1985). The dendrogram was constructed using the multiple sequence alignment algorithm ... Higgins and Sharp, 1988) and the unweighted pair-group method with arithmetic mean

DISCUSSION

We designed a PCR-based method for identification of mosquito-borne flaviviruses using two oligonucleotides corresponding to consensus sequences in their genome. These primers VD8 and EMF1 framed a sequence of 500-700 nucleotides containing variable regions suitable for genetic classification and analysis of the relationships among those viruses. Previous studies have proven the reliability of direct sequencing RT/PCR-derived flavivirus DNA fragments for molecular epidemiological studies (Deubel *et al.*, 1993; Lee *et al.*, 1992; Lewis *et al.*, 1992). Se-

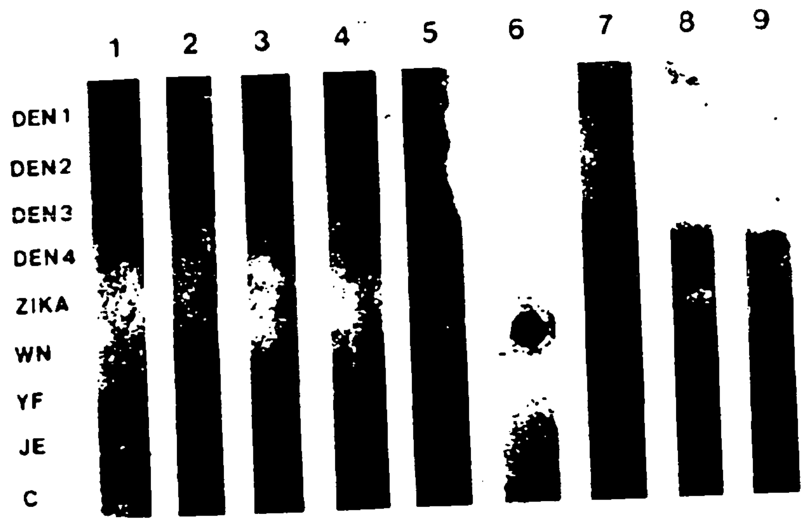

Fig. 4. Specificity of reverse hybridization assays.

Recombinant plasmids containing DEN1, DEN2, DEN3, DEN4, ZIKA, WN, YF and JE virus cDNA sequences and a plasmid without any flavivirus sequence were dotted onto nitrocellulose strips (C). Each strip was then hybridized with different PCR products labelled with digoxigenin. PCR products were obtained from RNA isolated from mosquitoes infected with dengue DEN1 (1), DEN2 (2), DEN3 (3), DEN4 (4), ZIKA (5), WN (6), YF (7) and JE (8) viruses or from mock-infected mosquitoes (9) using VD8 and EMF1 primers, and were labelled during the PCR by incorporation of dUTP-11 digoxigenin. The nitrocellulose strips were washed at 72°C. Products retained on the strips by hybridization to the plasmid were revealed by anti-digoxigenin antibodies labelled with alkaline phosphatase.

Table II. Comparison of the sensitivity of DEN2 virus RT/PCR to cell culture for flavivirus identification.

Number of PFU per assay (*)	250	50	5	0.5	Control
Hybridization test					
(ng probe/dot)					
100 ng	+ + +	+ + +	+	−	−
10 ng	+ +	+ +	−	−	−
1 ng	+	−	−	−	−
0.1 ng	−	−	−	−	

(*): virus samples ...

(**): Immunofluorescence for virus identification was performed ...

quence comparison of the flavivirus reference strains with other variants indicated a variability of between 0.5 and 22.3 % within individual serotypes. The highest variability was observed for the 3' non-coding region which had been subjected to deletions/insertions during evolution (table I). However, this region contains serocomplex- and serogroup-specific fragments which may be specifically recognized by cellular or viral proteins during virus replication (fig. 2). The sequence of the ZIKA virus was unknown. Although not assigned to a subgroup, it has been suggested to be most closely related to YF, Spondweni and Uganda S viruses, based on epidemiological and pathogenic characteristics (Monath, 1990). The sequences of the VD8-EMF1-amplified products suggest that this virus is well separated from all other viruses in the phylogenetic tree. Only one band corresponding to the amplified product was detected, but this does n[illegible] mean that a downstream repeat of the CS2 se[illegible]ence does not exist. Possibly, one of the seq[illegible]ces is involved in secondary structure preve[illegible] [illegible]ybridization, and this virus gives a single [illegible]nd.

The RT/PCR m[illegible]od has also been developed as an alternati[illegible] [illegible] tissue culture for rapid and sensitive detectic[illegible] f [illegible] viruses in mosquitoes and biological [illegible] [illegible]. Identification of flavivirus usually tak[illegible] re [illegible]an two weeks and requires specific m[illegible]clonal antibodies not available for all flavi[illegible]ses. Moreover, viral infectivity is preserv[illegible] [illegible] when samples are maintained in the co[illegible] [illegible]e accuracy of flavivirus PCR has been de[illegible]trated primarily using cell culture fluids or vir[illegible] seed lots, and only rarely using serum [illegible] mosquito specimens (Deubel *et al.*, 1990 [illegible]ille *et al.*, 1991; Lee *et al.*, 1992). The prese[illegible]t study confirms that RNA from infected mosqu[illegible]oes can be detected. Using universal primers coupled to specific hybridization, it is possible to detect more than one virus in multiply-infected patients (Gubler *et al.*, 1985; Laille *et al.*, 1991) and in field-caught mosquito pools. PCR is also able to detect multiple [illegible] species which may interfere with iden-

[illegible] Amplified DNA is usually identified either [illegible]

probing the amplified product with type-specific oligonucleotides (Henchal *et al.*, 1991; Lanciotti *et al.*, 1992), by using type-specific oligonucleotide primers in independent runs (Eldadah *et al.*, 1991; Tanaka, 1993; Trent and Chang, 1992) or by estimating the different size of the amplified products when a mixture of oligonucleotide primers, each specific for one virus serotype, is used (Morita *et al.*, 1991). All these tests use type-specific sequences for RT/PCR and identification which may be subject to variation within a serotype. The use of consensus sequences for RT/PCR coupled to spot hybridization with cloned DNA probes avoids this problem. We have previously demonstrated the advantage of non-radioactive cloned DNA probes compared to radiolabelled probes for identification of DEN-amplified target DNA (Deubel *et al.*, 1990). If every flavivirus probe were labelled, the number of different labelled probes required, and consequently the number of independent hybridization tests, would be very large. In order to circumvent these tedious steps, we inverted the hybridization protocol by dotting the cloned DNA probes on a single strip and by labelling cDNA during PCR. This easy-to-perform method facilitates the analysis of individual samples by reducing the number of hybridizations, as each strip is pre-coated with all the different flavivirus sequences. We had established in a previous test using direct hybridization of the amplified products with specific probes that RT/PCR was 20 times more sensitive than the cell culture for DEN virus identification (Deubel *et al.*, 1990). However, our present study showed that the same titre of virus was detected by RT/PCR and by cell culture for flavivirus diagnosis. Either the RT or the staining method for hybrid detection may account for this divergence. The suitability and validity of the method are currently being demonstrated on field samples.

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Identification de séquences nucléiques spécifiques des flavivirus transmissibles par les moustiques à l'aide d'amorces universelles et de la PCR

Nous avons développé une technique associant la transcription réverse et l'amplification en chaîne par polymérase (RT/PCR) pour la détection des flavivirus transmissibles par les moustiques. Le couple d'amorces correspond à deux séquences particulièrement conservées dans ces virus, l'une étant située vers la fin de la région 3' non codante et l'autre à environ 300 nucléotides avant la fin du gène de la protéine non structurale NS5. Nous avons analysé les séquences amplifiées entre ces amorces pour les virus de la dengue (sérotypes 1 à 4), de l'encéphalite japonaise, West Nile, de la fièvre jaune et Zika. L'étude comparative d'une fraction du gène NS5 codant pour 97 à 99 acides aminés selon le type viral indique une similitude de séquence nucléotidique comprise entre 56 et 76 %. Cette similitude n'est plus que de 20 à 36 % lorsque nous comparons les gènes correspondant à l'extrémité 3, non codante, dont la longueur peut varier de 190 à 491 nucléotides. L'étude comparative de la séquence nucléotidique du virus Zika avec celle des autres virus confirme qu'il n'est pas apparenté à d'autres flavivirus, comme cela avait été démontré dans la classification sérologique traditionnelle. Les plasmides recombinants contenant les séquences des huit flavivirus étudiés ont été utilisés dans un test d'hybridation moléculaire pour identifier le matériel génétique obtenus par RT/PCR de l'ARN viral extrait de moustiques infectés au laboratoire par injection intracoelomique. Ces plasmides ont été déposés sur une languette de nitrocellulose puis incubés avec le produit d'amplification marqué par la digoxigénine durant l'étape de PCR. Les plasmides contenant les gènes des quatre sérotypes de la dengue ont été partiellement délétés des séquences de la région NS5 pour augmenter la spécificité. Cette méthode spécifique et rapide représente une amélioration substantielle par rapport à notre précédente technique de RT/PCR pour la détection rapide de flavivirus dans les échantillons biologiques.

Mots-clés: Flavivirus, Séquençage, PCR, RT; Identification virale, Epidémiologie.

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An Integrated Target Sequence and Signal Amplification Assay, Reverse Transcriptase–PCR–Enzyme-Linked Immunosorbent Assay, To Detect and Characterize Flaviviruses

GWONG-JEN J. CHANG,* DENNIS W. TRENT, A. VANCE VORNDAM, EDGARDO VERGNE, RICHARD M. KINNEY, AND CARL J. MITCHELL

Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Public Health Service, Department of Health and Human Services, Fort Collins, Colorado 80522

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We previously described a reverse transcriptase-PCR using flavivirus genus-conserved and virus species-specific amplimers (D. W. Trent and G. J. Chang, p. 355–371, *in* Y. Becker and C. Darai; ed., Frontiers of Virology, vol. 1, 1992). Target amplification was improved by redesigning the amplimers, and a sensitive enzyme-linked immunosorbent assay (ELISA) technique has been developed to detect amplified digoxigenin (DIG)-modified DNA. A single biotin motif and multiple DIG motifs were incorporated into each amplicon, which permitted amplicon capture by a biotin-streptavidin interaction and detection with DIG-specific antiserum in a colorimetric ELISA. We evaluated the utility of this assay for detecting St. Louis encephalitis (SLE) viral RNA in infected mosquitoes and dengue viral RNA in human serum specimens. The reverse transcriptase-PCR-ELISA was as sensitive as isolation of SLE virus by cell culture in detecting SLE viral RNA in infected mosquitoes. The test was 89% specific and 95 to 100% sensitive for identification of dengue viral RNA in serum specimens compared with isolation of virus by *Aedes albopictus* C6/36 cell culture and identification by the indirect immunofluorescence assay.

Flaviviruses are arthropod-transmitted viruses that belong to the family *Flaviviridae* (1). Viruses in this family include the etiologic agents of dengue (DEN), yellow fever (YF), Japanese encephalitis (JE), West Nile encephalitis, Murray Valley encephalitis, St. Louis encephalitis (SLE), and tick-borne encephalitis (9). Conventional flavivirus diagnosis is based on isolation and identification of virus from clinical specimens or the presence of virus-specific antibody in the patient serum specimen (9). The exceptional sensitivity of the PCR permits a millionfold amplification of specific nucleotide sequences in vitro (14). This technique provides an attractive approach for rapid detection and identification of flaviviruses in mosquitoes and clinical specimens when virus cultivation is difficult or time consuming and when diagnosis impacts on clinical treatment and has implications for vaccination and mosquito control.

Several reverse transcriptase (RT)-PCRs using different pairs of primers (amplimers) for specific viruses have been developed for detecting flavivirus RNA. Identification of virus to species level has usually been accomplished by determining the size of amplified DNA by agarose gel electrophoresis (3, 6–8, 10) or by hybridization with labeled virus species-specific (VSS) probes (2, 6, 8). Agarose gel electrophoresis is more convenient but less sensitive than hybridization. A combination of nested PCR and agarose gel electrophoresis is more sensitive than hybridization for detecting and identifying the four serotypes of DEN virus (DEN1 through DEN4) (8). However, this technique is subject to amplicon contamination during the second cycle of amplification. We have described a simple strategy to detect medically important flaviviruses in a single-vessel RT-PCR (15). This assay consists of a flavivirus genus-conserved (FGC) amplimer pair (FUDJ9166 and CFDJ9977) and 11 individual VSS up amplimers that hybridize with specific sequences in the carboxyl-terminal one-third portion of the NS5 (nonstructural) gene. The FGC amplimer pair amplifies an amplicon of 830 bp in the NS5 gene of all flaviviruses tested (15). To determine the species of viral RNA, the FGC down amplimer (CFDJ9977) is used in combination with an individual VSS up amplimer. Amplicons of appropriate sizes are generated if the virus-specific up amplimer and viral RNA are homologous. This RT-PCR can also be used to identify previously unrecognized flaviviruses by nucleotide sequence analysis of the DNA amplified by the FGC amplimer pair (15).

To improve the sensitivity and specificity of the flavivirus RT-PCR, we have modified the FGC down amplimer, CFD2-4, and developed an integrated target and signal amplification assay. This assay employs RT-PCR in the presence of digoxigenin (DIG)-11-dUTP to amplify target sequences in the NS5 gene of the flavivirus RNA and uses the enzyme-linked immunosorbent assay (ELISA) to detect the DIG-modified amplicon DNA. Amplimers have been designed to detect and type the medically important flaviviruses in mosquitoes and serum specimens.

MATERIALS AND METHODS

...us strains and specimens... ...ection ... Division ... Vector-Borne Infectious Diseases, Centers ... Disease

...ne Infectious Diseases, ...llins, CO 80522. Phone ...03) 221-6447. Fax ...

Control and Prevention (CDC), Fort Collins, Colo. YF virus strain 17D and SLE virus strain MSI-7 were titrated in Vero cells (16).

Insectary-maintained *Culex pipiens* mosquitoes were inoculated intrathoracically with 0.5 PFU of SLE virus per 350 nl. Since mosquitoes are more susceptible than Vero cells for flavivirus replication, the virus dose used is sufficient to infect all inoculated mosquitoes. Sixteen uninfected mosquitoes were frozen at zero hour as the normal mosquito control. Two to four infected mosquitoes were removed and frozen at −70°C at 4.5, 18.5, 48, 96, and 144 h post inoculation (p.i.). Individual mosquitoes were homogenized separately in 1 ml of Eagle's minimal essential medium containing 5% bovine serum albumin and frozen at −70°C. The infection status of each mosquito was verified by plaque titration in Vero cells (16).

Serum specimens from DEN virus patients were obtained from the San Juan Laboratories, Division of Vector-Borne Infectious Diseases, CDC, San Juan, P.R. To evaluate the sensitivity and specificity of the RT-PCR-ELISA, we analyzed 91 serum specimens previously shown to contain DEN virus as demonstrated by virus isolation in *Aedes albopictus* C6/36 cell culture and virus identification by indirect immunofluorescence assay (IFA) (5). Twenty-four specimens negative by C6/36 cell culture and IFA were included as controls. All of the specimens were assigned a random code prior to blind testing by RT-PCR-ELISA.

RNA extraction. Nucleic acids were extracted from virus seed stocks, homogenized mosquito suspensions, or human serum specimens by the method described by Lanciotti et al. (8). The extracted nucleic acid was resuspended in distilled water and stored at −70°C.

Capture of flaviviral RNA from mosquitoes before RT-PCR. To remove nonspecific inhibitors of the RT-PCR, viral RNA in infected mosquito extracts was captured by using a modification of the method described by Lanciotti et al. (8). Oligonucleotide primer Bio-CFDJ9977 or Bio-CFD2-4 served as the flaviviral RNA capture probe. Nucleic acid extracted from homogenized mosquitoes was mixed with 5 pmol of capture probe in wash buffer (0.1 M Tris-HCl, pH 5.5; 0.1 M NaCl), heated at 94°C for 2 min, and slowly cooled to room temperature. Biotinylated probe (Bio-probe)–viral RNA complexes were captured by the addition of 10 μl of prewashed streptavidin-coated magnetic beads (M-280 streptavidin, binding capacity of 2 pmol/μl; Dynal, Inc., Great Neck, N.Y.). Following incubation at room temperature for 30 min, Bio-probe–viral RNA–M-280 complexes were immobilized during all washing steps with a magnetic particle concentrator (Dynal MPC-E; Dynal, Inc.). Bio-probe–viral RNA–M-280 complexes were washed three times with wash buffer to remove unbound components, and the complexes were resuspended in 20 μl of distilled H_2O. Captured viral RNA was separated from the complex by heating at 80°C for 2 min and quick chilling at 4°C. RNA in the supernatant was separated from the magnetic beads at 4°C and collected.

Synthesis of oligonucleotide amplimers. The FGC and VSS amplimers used in the RT-PCR-ELISA, except CFD2-4 and DEN4-9580, have been previously described (Table 1) (15). [illegible] oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer, model 380A, by using standard phosphoramidite chemistry (Applied Biosystems Inc., Foster City, Calif.). Biotin was attached to the 5' end of the CFDJ9977 and CFD2-4 amplimers by using biotin-on phosphoramidite chemistry (Clontech Laboratories, Inc., Palo Alto, Calif.). Synthetic oligonucleotides were purified by using OPC columns (Applied Biosystems, Inc.).

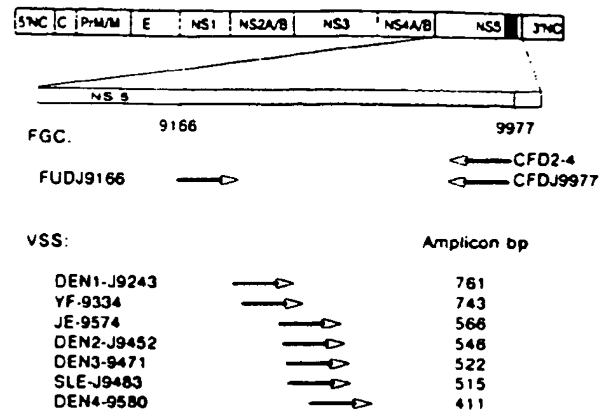


FIG. 1. Schematic diagram of the 11-kb flavivirus genome showing the relative locations of FGC and VSS amplimers. Arrows indicate the 5'-to-3' direction of amplimers. Amplimers specific for DEN1, DEN2, DEN3, DEN4, JE, SLE, and YF viruses are designated DEN1-J9243, DEN2-J9452, DEN3-9471, DEN4-9580, JE-9574, SLE-J9483, and YF-9334, respectively. NC, noncoding region; C, nucleocapsid protein; PrM/M, premembrane and membrane protein; E, envelope protein; NS, nonstructural protein.

RT-PCR as a target sequence amplification system. A single-vessel RT-PCR was used to convert target viral RNA to cDNA with subsequent amplification of the cDNA to double-stranded DNA. Reactions were done in a 25-μl volume that contained the following components: resuspended viral RNA extract (1 to 10 μl); 50 mM KCl; 10 mM Tris-Cl, pH 8.5; 1.5 mM $MgCl_2$; 0.01% (wt/vol) gelatin; 200 μM (each) the four deoxynucleoside triphosphates (Promega, Madison, Wis.); 2.5 mM dithiothreitol; 250 nM (each) (unless otherwise specified) up and down amplimers; 2.5 U of RAV-2 RT (Amersham Corp., Arlington Heights, Ill.) per 100 μl; and 2.5 U of Amplitaq polymerase (The Perkin-Elmer Corp., Norwalk, Conn.) per 100 μl. Reaction mixtures were incubated in a model 9600 Thermocycler (The Perkin-Elmer Corp.) for 1 h at 55°C to facilitate the RT reaction and for 4 min at 94°C for denaturation, after which they were subjected to 25 cycles of denaturation (94°C for 1 min), amplimer annealing (55°C for 1 min), and amplimer extension (72°C for 3 min), followed by a 10-min incubation at 72°C.

To incorporate DIG-dUMP directly into the amplicon, reactions were performed as described above with the addition of 10 μM DIG-11-dUTP (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in the reaction mixture. Although a higher concentration of DIG-11-dUTP in the reaction mixture resulted in increased optical density (OD) readings in ELISAs, test sensitivity was not significantly improved (data not shown).

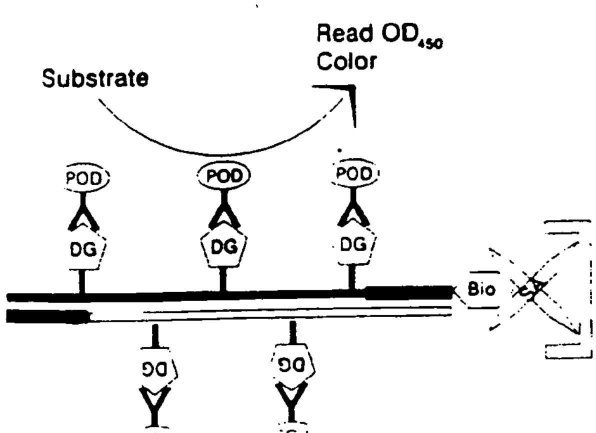
ELISA amplification system. DIG-modified amplicons (DIG-amplicons) were characterized by agarose gel electrophoresis and ELISA. Five microliter portions of reaction [illegible] NA was stained with ethidium bromide. Because individual VSS amplimers primed at different positions in the NS5 gene of the viral RNA, the amplicon size for each flavivirus was different (Fig. 1 and Table 1).

TABLE 1. Amplimers used in RT-PCR-ELISA identification of flaviviruses

Amplimer type and designation	Oligonucleotide sequence (5' to 3')	Amplicon size (bp)
FGC amplimers		
Bio-CFD2-4[a]	5'-TTTGAGCATGTCTTCCGTCGTCATCC	
Bio-CFDJ9977[a]	5'- GCATGTCTTCCGTCGTCATCC	
FUDJ9166[b]	5'-GATGACACAGCAGGATGGGAC	838 or 832[c]
VSS up amplimers		
DEN1-J9243	5'-GCCTGAACATGCTCTATTGGCT	761
DEN2-J9452	5'-TCTTCAAAAGCATTCAGCACCT	546
DEN3-9471	5'-CCCATCCGCTAGAGAAGAAAATTACAC	522
DEN4-9580	5'-GGTTTGGCACTTCCCTCCTCTTCTTG	411
JE-9574	5'-GACCACAACACTTGGAACAGCTAC	566
SLE-J9483	5'-ACGATTGGCCAAAGCGGTTGAG	515
YF-9334	5'-ACAAGCAGTGATGGAAATGACA	743
Nested-PCR amplimers[d]		
D1	5'-TCAATATGCTGAAACGCGCGAGAAACCG	
D2	5'-TTGCACCAACAGTCAATGTCTTCAGGTTC	511
TS1	5'-CGTCTCAGTGATCCGGGGG	482
TS2	5'-CGCCACAAGGGCCATGAACAG	119
TS3	5'-TAACATCATCATGAGACAGAGC	290
TS4	5'-CTCTGTTGTCTTAAACAAGAGA	392

[a] Down amplimer.
[b] Up amplimer.
[c] Amplicon size obtained by using Bio-CFD2-4 or Bio-CFDJ9977, respectively.
[d] Reported by Lanciotti et al. (8).

A schematic representation of the ELISA detection protocol is shown in Fig. 2. DIG-amplicons were diluted in a fourfold dilution series with rinse buffer (phosphate-buffered saline with 0.05% Tween 20). Fifty-microliter aliquots of the diluted DIG-amplicons were added to each well of a 96-well ELISA plate (Immulon II; Dynatech Laboratories Inc., Chantilly, Va.) that had previously been coated with streptavidin (1 μg per well; Boehringer Mannheim Biochemicals) in 100 μl of coating buffer (Na_2CO_3-$NaHCO_3$, pH 9.6) and blocked with 100 μl of blocking buffer (3% normal goat serum in rinse buffer) per well. Biotinylated DIG-amplicons were bound to the microtiter plate through biotin-streptavidin interaction by incubation at 37°C for 1 h. The plates were washed three times with rinse buffer, followed by the addition to each well of 50 μl of rinse buffer containing 5 mU of horseradish peroxidase-conjugated sheep anti-DIG Fab fragment (Boehringer Mannheim Biochemicals), incubation at 37°C for 1 h, and three washes with rinse buffer. One hundred microliters of the enzyme substrate-color indicator (0.01% 3,3',5,5'-tetramethyl benzidine, 0.005% H_2O_2 in 0.1 M citrate-acetate buffer, pH 6.0) was added to each well, and the plates were incubated at room temperature for 10 min. Reactions were stopped by the addition of 50 μl of 2 M H_2SO_4 per well, and the OD at 450 nm (OD_{450}) was measured in each well.



[illegible] amplicons were captured [illegible] streptavidin [illegible] and were detected by using horseradish peroxidase-conjugated sheep anti-DIG Fab fragment (POD) in a standard ELISA format. The biotin moiety was from biotinylated FGC amplimers, Bio-CFD2-4

Development of testing algorithms for routine diagnosis of flavivirus infection. A panel of 115 randomly coded serum specimens, including 91 positive and 24 negative by virus isolation in C6/36 cells and IFA, was used in a blind test to determine the sensitivity and specificity of the RT-PCR-ELISA as a diagnostic procedure for DEN virus infection. Results were recorded as flavivirus negative, flavivirus positive (with DEN virus serotype), or virus species undetermined (Fig. 3). Specimens that gave disparate results were reevaluated by virus isolation in C6/36 cells and IFA (5) and nested PCR (8).

RESULTS

[illegible] ELISA signal amplification system. [illegible]

[illegible] amplimers used to prime synthesis [illegible] specific amplicons during the RT-PCR. Although nonspecific target amplification can be decreased or eliminated by raising the amplimer annealing temperature from 50 to 55°C, some nonspecific amplimer-RNA interaction can occur at higher

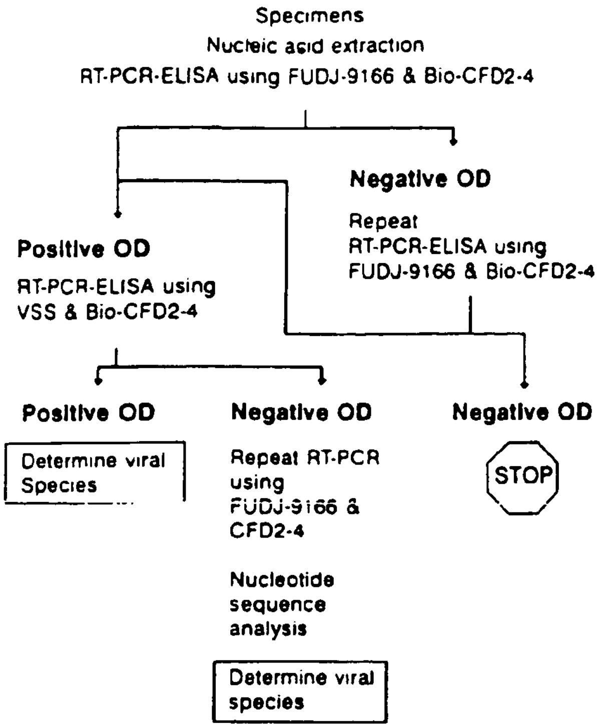


FIG. 3. Test algorithm for RT-PCR-ELISA detection of flavivirus RNA in clinical specimens.

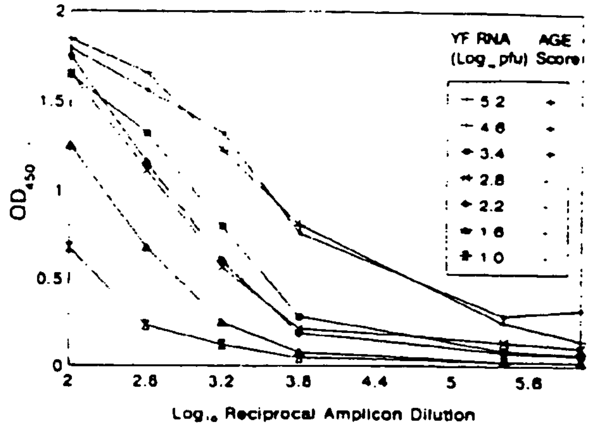
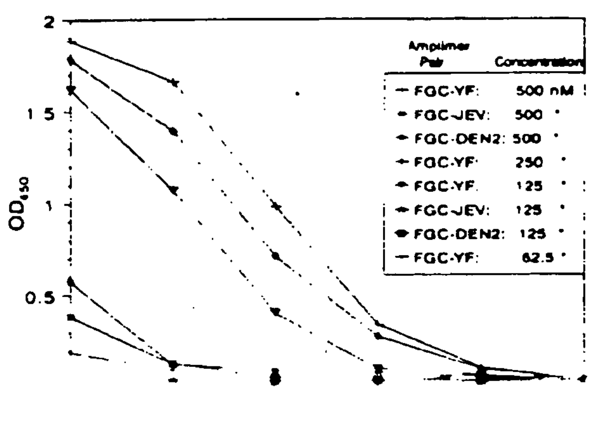


FIG. 5. Sensitivity of the RT-PCR-ELISA for detection of YF viral RNA. Assays were performed with amplimers Bio-CFD2-4 and YF-9334 at 250 nM for 25 PCR cycles. Colorimetric ELISA detection (graphs) of the amplicon is compared with visual detection of the ethidium bromide-stained amplicon analyzed by agarose gel electrophoresis (AGE score; + or −) following RT-PCR using a 1.0 to 5.2 $\log_{10}$ PFU equivalent of YF viral RNA.

amplimer concentrations and result in a higher nonspecific OD_{450}. To illustrate the effect of amplimer concentration on ELISA specificity, different concentrations (500, 250, 125, and 62.5 nM) of down FGC amplimer CFD2-4 and up VSS amplimer YF-9334, DEN1-J9243, DEN2-9452, DEN3-9471, DEN4-9580, or JE-9574 (Table 1) were used to detect a 6.2 $\log_{10}$ PFU equivalent of YF viral RNA (Fig. 4).

Down amplimer Bio-CFD2-4 paired with heterologous VSS up amplimers DEN2-J9452 and JE-9574 at a concentration of 500 nM gave unacceptably high OD_{450}s of 0.58 and 0.38, respectively, at an amplicon dilution of 1:100 (Fig. 4). Background ODs were below 0.05 when 250, 125, or 62.5 nM heterologous VSS amplimers were used in RT-PCRs (Fig. 4). The homologous Bio-CFD2-4–YF-9334 amplimer pair at concentrations of 500, 250, and 125 nM resulted in similar OD_{450}s of 1.62 to 1.88 at the 1:100 dilution of DNA amplified from YF viral RNA (Fig. 4). Amplicon yields were nearly equal when this amplimer pair was used at concentrations of 500 and 250 nM but decreased by about 80% at the 125 nM concentration (data not shown). At an amplimer concentration of 62.5 nM, amplicons were undetectable by agarose gel electrophoresis (not shown) but were weakly positive by ELISA (Fig. 4). Experiments using heterologous and homologous amplimers with DEN1, DEN2, DEN3, DEN4, or JE viral RNAs gave similar results (data not shown). On the basis of high amplicon yield and low nonspecific reactivity, an amplimer concentration of 250 nM was selected for use throughout the remainder of this study.

Sensitivity of RT-PCR-ELISA for detecting viral RNA. YF viral RNA was used to illustrate the sensitivity of the RT-PCR-ELISA as an integrated amplification and detection system. Various amounts of virus RNA, equivalent to 5.2, 4.6, 3.4, 2.8, 2.2, 1.6, or 1.0 $\log_{10}$ PFU of YF virus, and 250 nM amplimer pair Bio-CFD2-4–YF-9334 were used. The relative sensitivities of the RT-PCR-ELISA and the agarose gel electrophoresis-ethidium bromide staining methods were compared for detection of DIG-amplicons amplified from various amounts of YF viral RNA (Fig. 5). The RT-PCR-ELISA enabled detection of a 10-PFU equivalent of YF viral RNA (Fig. 5), in contrast to agarose gel electrophoresis, with which a specific amplicon band was visible only when greater than a [illegible] $\log_{10}$ PFU equivalent [illegible] RNA was used (Fig. [illegible]). Thus, ELISA was at least [illegible] more sensitive than agarose gel electrophoresis-ethidium bromide staining for detection of DIG-amplicons.



[illegible] amplimer concentration [illegible] RT-PCR-ELISA. Bio-CFD2-4 (FGC) was paired with different VSS amplimers, YF-9334 (YF), JE-9574 (JEV), and DEN2-J9452 (DEN2) in an RT-PCR-ELISA to detect YF viral RNA. Amplimer pairs were tested at 500, 250, 125, and 62.5 nM.

Viral RNA detection in mosquitoes infected with SLE virus. Application of the RT-PCR-ELISA to detect viral RNA in flavivirus-infected mosquitoes was investigated by analyzing

TABLE 2. Comparison between virus isolation and RT-PCR-ELISA in detection of SLE virus-infected mosquitoes

Time (h) p.i.	No. of specimens tested	Result by:		
		Virus isolation[a]	RT-PCR-ELISA[b]	
			Uncaptured RNA[c]	Captured RNA[d]
2.5	2	−, −	−, −	−, −
18.5	4	−, −, −, −	−, −, −, −	−, 2.6, −, 2.6
48.0	3	1.7, 2.4, 2.2	−, −, 2.6	2.6, 2.6, 2.6
96.0	3	3.4, 4.8, 5.4	3.2, 3.8, >4.4	3.2, 3.8, >4.4
144.0	4	5.7, 4.6, 5.6, 5.9	>4.4, 3.8, >4.4, >4.4	>4.4, 3.8, >4.4, >4.4
0 (uninfected controls)	16	− (all)	<2.0 (all)[e]	<2.0 (all)

[a] −, negative result. Numbers indicate $\log_{10}$ PFU/ml measured by plaque titration in Vero cells.
[b] −, negative result. Numbers indicate $\log_{10}$ reciprocal endpoint titers determined by ELISA.
[c] Nucleic acid extracted from mosquito homogenates was used directly in RT-PCR-ELISA.
[d] Virus-specific RNA purified from nucleic acid extracts by Bio-probe–streptavidin capture.
[e] All uninfected control mosquitoes had OD_{450}s of 0.1 to 0.25 at a 1:100 amplicon dilution.

mosquitoes inoculated with SLE virus. Mosquitoes were assayed for infectious virus by plaque titration in Vero cell monolayers and for viral RNA by RT-PCR-ELISA at various times p.i. SLE virus could be isolated from mosquitoes 48 to 144 h p.i. (Table 2). Only one of the three mosquitoes tested at 48 h p.i. was positive by RT-PCR-ELISA when total nucleic acid was used in the test without purification of the viral RNA. However, following capture and elution from probe-magnetic bead complexes, viral RNA was detected by RT-PCR-ELISA in all of the mosquitoes tested at 48 to 144 h p.i., as well as in two of four mosquitoes tested at 18.5 h p.i. (Table 2). Although detection of SLE viral RNA in mosquitoes by RT-PCR-ELISA without RNA capture was less sensitive than virus detection by isolation of virus in cell culture, RT-PCR-ELISA of captured SLE viral RNA was at least as sensitive as virus isolation (Table 2).

RT-PCR-ELISA for routine detection and typing of DEN viruses from viremic human serum. To determine the sensitivity and specificity of the RT-PCR-ELISA as a diagnostic test, a panel of 115 randomly coded human serum specimens was analyzed. Ninety-one of the serum specimens had previously been shown to contain DEN virus by virus isolation and IFA in C6/36 cell cultures (5). By the testing algorithm shown in Fig. 3, 97 of the serum specimens were positive for DEN viral RNA by RT-PCR-ELISA. A comparison of the PCR results with those obtained by virus isolation and IFA revealed discordance for 14 specimens, as shown in Table 3. These 14 specimens were again tested by virus isolation and IFA in C6/36 cell culture. By virus isolation and IFA, specimens 17, 30, 93, 107, 124, 163, 187, and 200, which were initially reported as negative, were positive for DEN2 virus on retrial (Table 3). The remaining six discordant specimens (8, 15, 48, 91, 135, and 180) were retested by RT-PCR-ELISA as well as by the nested-PCR technique (8). Specimens 91 and 135, which were identified by virus isolation and IFA as positive for DEN3 and DEN4 virus, [illegible] positive for DEN2 and DEN3 viral RNA, respec- [illegible] by IFA but as DEN2 positive by RT-PCR-ELISA and nested PCR. Specimens 15 and 48 were DEN1 positive and DEN2 positive by IFA but were both negative by RT-PCR-ELISA and nested PCR. Specimens 8 and 180 were recorded as false negative by IFA, whereas specimens 15 and 48 were [illegible] negative by RT-PCR-ELISA.

[illegible] comparisons were made between [illegible] RT-PCR-ELISA results and those obtained by virus isolation and IFA (Table 4). Both methods provided 89% overall specificity and 98% overall sensitivity in the detection of specimens containing DEN virus or viral RNA. Both methods showed ~~95 to 100%~~ sensitivity in the identification of human serum specimens infected with each of the four serotypes of DEN virus (Table 4). Identification of DEN virus or viral RNA in these clinical specimens by RT-PCR-ELISA was comparable to the virus isolation-IFA method in terms of specificity and sensitivity. However, RT-PCR-ELISA offers the advantage of speed in diagnostic testing.

DISCUSSION

The epidemiology and clinical diagnosis of flaviviruses, which are important causes of human disease, are major public health concerns (9). The RT-PCR amplification of viral RNA provides a rapid and specific technique for the detection and identification of flaviviruses and is as sensitive as virus isolation in cell cultures and in mice (2, 3, 6–8, 10). Because several flaviviruses may be transmitted by the same

TABLE 3. Repeat testing of 14 human serum specimens for the presence of DEN virus (C6/36 cell culture and IFA) or DEN viral RNA (RT-PCR)

Specimen no.	Serotype identified by:			
	C6/36 cell culture and IFA		RT-PCR	
	Preliminary	Retrial	ELISA	Nested[a]
8	N[b]	N	2	2
15	1	1	N	N
17	N	2	2	ND[c]
30	N	2	2	ND
48	2	N	N	N
91	3	N	2	2
93	N	2	2	ND
[illegible]	[illegible]	[illegible]	[illegible]	ND
187	N	2	2	ND
200	N	2	2	ND

[a] The nested RT-PCR was performed as described by Lanciotti et al. (8).
[b] N, negative specimen.
[c] ND, not done.

TABLE 4. Specificity and sensitivity of IFA or RT-PCR-ELISA to detect DEN virus or viral RNA from human serum specimens

Result and/or serotype	No. identified by IFA/no. identified by PCR[a]	No. identified by PCR/no. identified by IFA[b]	Specificity[c]		Sensitivity[d]	
			IFA	PCR	IFA	PCR
Positive						
DEN	97/95	97/95	89	89	98	98
DEN1	26/25	25/25			96	96
DEN2	36/35	37/35			97	95
DEN3	10/10	10/10			100	100
DEN4	25/25	25/25			100	100
Negative	16/14	16/14				
False positive	2 (IFA)	2 (PCR)				
False negative	2 (IFA)	2 (PCR)				

[a] Number of specimens identified by IFA/number of the same specimens confirmed by PCR.
[b] Number of specimens identified by PCR/number of the same specimens confirmed by IFA.
[c] Specificity of assay = no. of negative specimens/(no. of negative specimens + no. of false-positive specimens) × 100.
[d] Sensitivity of assay = no. of positive specimens/(no. of positive specimens + no. of false-negative specimens) × 100.

species of mosquito in the same geographic area at the same time, the isolation of more than one virus from mosquitoes or clinical specimens is a possibility. By FGC- and VSS-amplimer-mediated RT-PCR (15), the RNA species of 11 medically important flaviviruses can be determined by the specific size of amplified DNA. This technique is also capable of identifying a previously uncharacterized or newly emerged flavivirus by use of the FGC amplimer pair and direct nucleotide sequence analysis of the amplified DNA (15).

Oligonucleotide probe hybridization (2, 6, 8) and reamplification with internal amplimers (8) utilize labeled probes or reamplification to increase the sensitivity and specificity of detection. These methods are more sensitive than agarose gel electrophoresis but are time-consuming and technically demanding. Detection and characterization of flaviviral RNA by the integrated target sequence (RT-PCR) and signal (ELISA) amplification system are accomplished by using the previously defined FGC and VSS amplimer sequences with some modifications (Table 1) (15). The Bio-CFD2-4 amplimer and DIG-11-dUTP used in the RT-PCR incorporate a biotin molecule and multiple DIG residues into the amplicon. This technique uses biotin-streptavidin as a separation system and DIG-anti-DIG as an indicator system to achieve a high level of sensitivity and technical simplicity. We detected as little as a 10-PFU equivalent of YF viral RNA, ~~indicating this method is as sensitive as the nested-PCR method of Lanciotti et al. (8).~~

As with PCR detection of other RNA viruses, sensitivity is largely determined by the efficiency of reverse transcription of the flavivirus genomic RNA in producing cDNA prior to PCR (13). False-negative RT-PCR results were obtained when DEN viral RNA isolated from DEN virus-infected mosquitoes was used in the PCR (8). Lanciotti et al. (8) speculated that an inhibitory component present in the mosquito extract inhibited the PCR. We have developed a method that facilitates capture of flaviviral RNA prior to [illegible] RT-PCR prior to virus isolation (Table [illegible]) suggests that the RNA capture procedure does remove nonspecific inhibitors and concentrates the viral RNA.

The RT-PCR-ELISA was equivalent to the standard virus isolation and IFA protocol in terms of test specificity (89%) and sensitivity (95 to 100%) in identifying DEN virus or viral RNA in clinical human serum specimens (Table 4). Six discordant specimens (Table 3) gave identical results when they were retested by RT-PCR-ELISA and nested PCR (8) (Table 3). Specimen 15, from which DEN1 virus was reisolated, was the only sample classified by the two PCRs as negative. Specimen 48, initially reported to be positive, was negative by the two PCRs and virus reisolation, suggesting that the virus had been inactivated and the RNA had been degraded. Results obtained by two different RT-PCR techniques for two indeterminate specimens, 91 and 135, differed from the initial results obtained by virus isolation and IFA. These discrepancies probably resulted from misinterpretation of the initial IFA. Unfortunately, we could not reisolate virus from these two specimens. Specimens 8 and 180 were positive by two different PCR techniques but negative by virus isolation, suggesting that inactive virus was present in these sera. Virus-antibody complexes or inactivation of viral infectivity during manipulation of the specimen may contribute to failure to isolate virus.

The RT-PCR for detection of flavivirus RNA is approximately 100 times more sensitive than an antigen capture assay (16). A major advantage of RT-PCR is that it can detect multiple flavivirus species in a single test, whereas antibody capture assays are virus specific. The technique we have developed has several advantages over other flavivirus PCR techniques and conventional methods. The entire method can be completed within 24 h, in contrast to the several days required to culture and perform immunologic identification. A single amplification with FGC amplimers is required to detect flavivirus RNA. A second test uses VSS amplimers to identify virus. Unlike amplimers that are located in virus structural genes, which are subject to more intense selection pressures, amplimers designed for this study hybridize with the highly conserved NS5 gene (11), which encodes a postulated viral RNA polymerase (14). [illegible] sequence analysis of amplicons generated by the [illegible] identity [illegible] viruses [illegible] construct new [illegible] amplimers [illegible] facilitate virus identification in RT-PCR. The ELISA format for detection of amplicons offers increased sensitivity and is more reliable than the hybridization or nested-PCR methods.

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Rapid Identification of Dengue Virus Serotypes by Using Polymerase Chain Reaction

KOUICHI MORITA, MARIKO TANAKA, AND AKIRA IGARASHI*

Department of Virology, Institute of Tropical Medicine, Nagasaki University, 12-4 Sakamoto-machi, Nagasaki, Japan 852

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Four primer pairs were selected on the basis of the published sequence data of four dengue virus serotypes so that each unique target sequence size could be amplified for each serotype by polymerase chain reaction. The procedure consists of (i) RNA preparation, (ii) reverse transcription, and (iii) polymerase chain reaction, all of which could be completed within 2 h in a single tube for each specimen. The amplified sequence size revealed by ethidium bromide-stained agarose gel electrophoresis was unique for each serotype, using infected culture fluid of isolates from dengue fever or dengue hemorrhagic fever patients in Thailand, Indonesia, and the Philippines as well as from prototype viruses, thus facilitating simultaneous identification and typing.

…engue viruses of four different serotypes (mosquito-…ne flaviviruses) are causative agents of dengue fever and …gue hemorrhagic fever, which are highly prevalent in …pical countries, especially those of Southeast Asia (6). …e identification and typing of dengue virus isolated from …ld-caught mosquitoes and from clinical specimens are …portant for epidemiological and clinical investigations. …velopment of type-specific dengue virus monoclonal an-…odies has greatly facilitated these steps (8, 9), but it is still …ne-consuming. In this study, we developed a simple and …pid method for the detection, identification, and typing of …ngue virus isolates by using polymerase chain reaction …CR), a technique for DNA amplification in vitro (16, 18).

MATERIALS AND METHODS

Virus strains. The dengue virus strains used in this study …e listed in Table 1 with their serotype, country of isolation, …ar of isolation, and clinical diagnosis. The viruses were …oculated once to C6/36 cells (10) in tube culture. Infected …uids were harvested 1 week after incubation at 28°C and …ored at −80°C until use.

Primers. All primers were synthesized with an Applied …iosystems DNA synthesizer (model 380B) and confirmed …r purity by ion-exchange gel chromatography (Gen-pack; …aters). When >1% of an incomplete length of oligomer …as observed, the primer was discarded and the sequence …as newly synthesized. The nucleotide sequence and refer-…nce information of the primers are listed in Table 2.

RT-PCR. (i) Rapid RT-PCR. Five microliters of infected …uid was incubated with an equal volume of detergent mix …1% Nonidet P-40, 10 U of RNase inhibitor [Takara Co., …yoto, Japan] in phosphate-buffered saline [−]) in a 500-μl …ppendorf-type tube for 1 min at room temperature. This …as followed by the addition of 90 μl of reverse transcription …RT)-PCR mix (100 pmol of each primer, 0.2 mM deoxynu-…leoside triphosphate, 10 mM Tris [pH 8.9], 1.5 mM $MgCl_2$, …0 mM KCl, 0.5 mg of bovine serum albumin per ml, 0.1% …odium cholate, 0.1% Triton X-100, 10 U of reverse tran-…criptase [Life Science Inc.] and 2 U of Tth DNA polymer-… [illegible] (Toyobo Co., Osaka … mineral oil, and the tube was incubated for 10 min at 53°C for RT. PCR amplification (92°C for 60 s, 53°C for 60 s, and 72°C for 60 s by thermal cycler; Iwaki Co., Tokyo, Japan) was started immediately after the RT and repeated 25 to 35 times. Five microliters of PCR product was subjected to agarose gel electrophoresis, and amplified DNA fragments were visualized by ethidium bromide staining.

(ii) Standard RT-PCR used in Fig. 1. Five microliters of RNA template was heated at 95°C for 3 min and added with 5 μl of complementary primer (100 pmol) in a 500-μl Eppendorf-type tube. The mixture was cooled down to 42°C and incubated for 5 min at that temperature. This was followed by the addition of 40 μl of RT mix (0.2 mM deoxynucleoside triphosphate, 10 mM Tris [pH 8.9], 1.5 mM $MgCl_2$, 80 mM KCl, 0.5 mg of bovine serum albumin per ml, 10 U of reverse transcriptase). The mixture was incubated at 53°C for 10 min for RT followed by the addition of 50 μl of the following mixture: 100 pmol of sense primer, 0.2 mM deoxynucleoside triphosphate, 10 mM Tris [pH 8.9], 1.5 mM $MgCl_2$, 80 mM KCl, 0.5 mg of bovine serum albumin per ml, 0.2% sodium cholate, 0.2% Triton X-100, and 2 U of Tth DNA polymerase. The reaction mixture was covered by 2 drops of mineral oil, and the PCR incubation was performed as described for the rapid RT-PCR procedure.

Dot blot hybridization. Ten microliters of each PCR product was heat denatured at 100°C for 5 min and mixed with 10 μl of 2 M ammonium acetate. The mixture was blotted on a Hybond N membrane (Amersham), using a Bio-Dot apparatus (Bio-Rad). The membrane was washed once with 1 M ammonium acetate, soaked in alkali solution (0.5 M NaOH, 1.5 M NaCl) for 10 min and neutralized in 0.5 M Tris HCl (pH 7.5)–1.5 M NaCl solution for 5 min. The membrane was dried and irradiated by UV light (256 nm) for 3 min.

The membrane was prehybridized with hybridization buffer containing 45% formamide (supplied by Amersham) at 42°C for 1 h. Molecularly cloned cDNA (50 ng) for each dengue virus serotype, containing the target nucleotide sequences, was labeled with ^{32}P-dCTP by using a nick translation labeling kit (Takara Co.) in accordance with the manufacturer's protocol. The radiolabeled probe was added … [illegible] … the membrane was incu-

TABLE 1. Information on dengue virus strains

Strain	Type	Country	Yr	Diagnosis[a]
D1 (Hawaiian)	D1	Prototype		
D2 (New Guinea B)	D2	Prototype		
D80-709	D2	Thailand		
PhMH10-84	D2	Philippines	1984	
D80-753	D2	Thailand	1980	
D3 (H87)	D3	Prototype		
U20-82	D3	Indonesia	1982	FUO
16-82	D3	Indonesia	1982	DHF
ThCMP15-82	D3	Thailand	1982	DHF
16562	D3	Philippines	1964	DHF
PhMH2311-83	D3	Philippines	1983	
PhMH4-84	D3	Philippines	1984	
V11	D3	Indonesia	1976	DF
D4 (H241)	D4	Prototype		
D80-785	D4	Thailand		
SI-YO SMB10	D4	Thailand	1978	DHF
No. 17	D4	Sri Lanka	1978	

[a] FUO, fever of unknown origin. DHF, dengue hemorrhagic fever. DF, dengue fever.

FIG. 1. Photograph of an agarose gel stained with ethidium bromide, containing PCR products obtained by four different procedures and using the dengue virus type 2 primer pair (D2S and D2C). (Lane 1) Purified RNA prepared from 5 μl of infected fluid (5 × 10⁵/ml) was heat denatured and annealed with complementary primer (D2C) at 42°C for 5 min and reverse transcribed for 10 min at 53°C, as described in Materials and Methods. After RT, PCR mix containing sense primer and Tth DNA polymerase were added (final volume, 100 μl) and amplified. (Lane 2) The same volume of infected fluid treated with Nonidet P-40 and RNase inhibitor, as described in Materials and Methods, was used as template and treated as in lane 1. (Lane 3) Purified RNA was used instead of infected fluid. The rest of the procedure is the same as described for lane 4. (Lane 4) Rapid RT-PCR was performed as described in Materials and Methods. All PCR amplifications were done 25 times.

sodium dodecyl sulfate for 10 min at room temperature once and at 42°C for 15 min three times. Then the membrane was dried and exposed to Amersham hyperfilm with intensifying screen at −80°C overnight.

RESULTS

Simplification of the RT-PCR procedure. To simplify the RT-PCR procedure, four different procedures were compared (Fig. 1). The lane 1 method is a standard RT-PCR procedure in which RNA is purified by phenol-chloroform extraction and heat denatured, followed by annealing of complementary primer (D2C) and RT prior to the PCR cycle. This procedure involves at least two handlings and incubations of tubes in a water bath prior to the PCR cycle. In lane 2, detergent-treated infected fluid is used as template instead of the purified RNA used in the lane 1 procedure. Lane 4, or rapid RT-PCR, was performed as described in Materials and Methods, using 5 μl of infected fluid (5×10^5 PFU/ml). The results of rapid RT-PCR, using purified RNA as template, are displayed in lane 3.

An amplified target DNA band of rapid RT-PCR with purified RNA as template (lane 3) showed a density as good as that in the standard RT-PCR procedure (lane 1). Furthermore, infected fluid treated by detergent was just as good a template as purified RNA in rapid RT-PCR (lane 3 and 4). All procedures could be completed within 2 to 3 h.

Sensitivity of rapid RT-PCR (Fig. 2). Infected fluid of dengue virus type 2 was diluted 10-fold with Eagle minimum essential medium, and 5 μl of each diluted sample was subjected to the rapid RT-PCR procedure, using primers D2S and D2C to evaluate the detection limit. After 25 cycles, the amplified target DNA band was detected in 1:100-diluted samples, or approximately 25 PFU of virus in each reaction tube (Fig. 2a). By 35-cycle amplification, positive results were obtained from 1:1,000-diluted samples, corresponding to 2.5 PFU/reaction tube (Fig. 2b). Thus, the sensitivity of rapid RT-PCR, using infected fluid, is sufficient to detect dengue virus genome in infected fluids of 500-PFU/ml virus titer.

Serotype specificity of each primer pair. The sequences o

TABLE 2. Nucleotide sequences of dengue virus primers

Code[a]	Sequence	Position	Reference(s)
[illegible]	[illegible]	2229–2248	15
[illegible]	[illegible]	[illegible]	[illegible]
D3C	TCCATTCTCCCAAGCGCCTG	[illegible]	[illegible]
D4S	CCATTATGGCTGTGTTGTTT	3973–3992	14, 20
D4C	CTTCATCCTGCTTCACTTCT	4370–4351	14, 20

[a] S, sense primer; C, complementary primer; D1, 2, 3, 4, dengue virus [illegible] and 4.

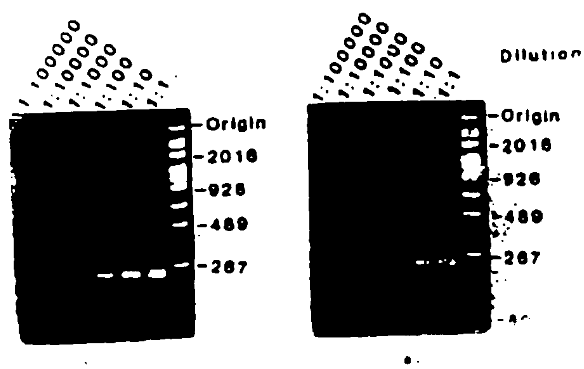

FIG. 2. Sensitivity [illegible]. Infected fluid was serially diluted in 10-fold steps [illegible] virus type 2 [illegible] minimum essential medium containing 10% fetal calf serum [illegible] subjected to rapid RT-PCR as described in Materials and Methods. The virus amount (1:1) was about 2.5×10^3 PFU in one reaction tube. (a) Amplified 25 PCR cycles; (b) amplified 35 PCR cycles.

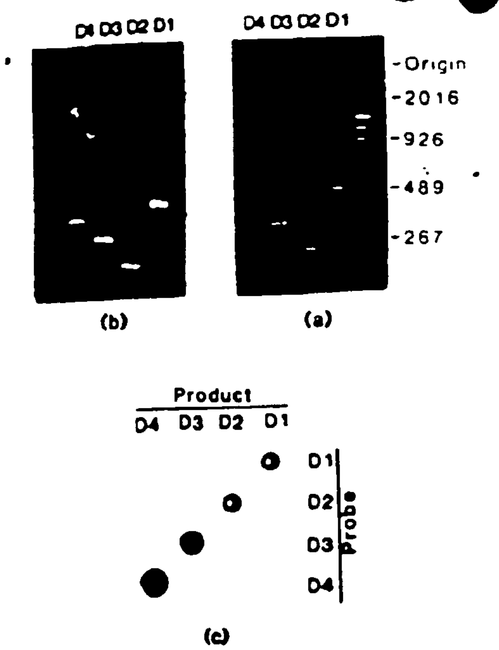

FIG. 3. Identification of dengue virus serotype by dengue virus-
:cific primer pairs. Infected fluid (5×10^5 to 8×10^7 PFU/ml) of
:h dengue prototype virus was mixed with detergent mix contain-
:(a) only homologous primer pairs (100 pmol each) or (b) four sets
primers (D1S and D1C, D2S and D2C, D3S and D3C, and D4S
d D4C, 100 pmol each) and subjected to the RT-PCR procedure as
scribed in Materials and Methods. D1, dengue virus type 1; D2,
ngue virus type 2; D3, dengue virus type 3; D4, dengue virus type
rototype viruses. Dengue virus types 1, 2, 3, and 4 show unique
igths of amplified DNA fragment: 490, 230, 320, and 398 bp,
spectively. (c) Dot blot hybridization with cloned cDNA probe of
ch dengue virus serotype. The PCR product of each dengue virus
pe in panel b was dotted on the membrane and hybridized with
:ch serotype-specific radiolabeled probe, as described in Materials
id Methods.

engue virus serotype-specific primer pairs were selected om published data (3, 4, 14, 15, 17, 20) by computer nalysis. Each primer pair reacted well to its homologous irus, and the size of each amplified DNA corresponded losely to the expected size (490, 230, 320, and 398bp for D1,)2, D3, and D4, respectively), which was calculated from he nucleotide sequences (Fig. 3a). The sensitivities of D1,)3, and D4 primer pairs are 8, 3.0, and 3.5 PFU/reaction ube, respectively.

All four primer pairs were then mixed and examined to letermine whether or not heterologous primers create a ackground DNA band in addition to the target DNA band. Figure 3b shows that only the target DNA fragments were amplified even when four sets of primers were present in the RT-PCR reaction mixture, indicating a high specificity of each primer pair for its homologous serotype.

We investigated the cross-reactivity of these four primer pairs to other flaviviruses. Dengue type 1, Japanese encephalitis, St. Louis encephalitis, Murray Valley encephalitis, and West Nile viruses were examined, using $>10^5$ PFU of each virus per tube in the reaction mixtures. No obvious ... other flaviviruses after 35 PCR

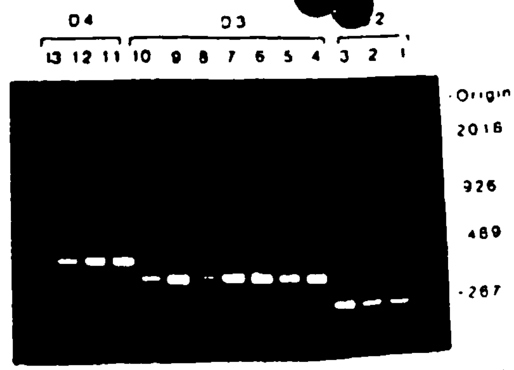

FIG. 4. Photograph of an agarose gel stained with ethidium bromide, demonstrating the serotype specificity of the four primer pairs among Southeast Asian dengue virus strains. The results are in complete agreement with serological results. Lanes: 1, strain D80-709; 2, PhMH10-84; 3, D80-753; 4, U20-82; 5, 16-82; 6, ThCMP15-82; 7, 16562; 8, PhMH2311-83; 9, PhMH4-84; 10, V11; 11, D80-785; 12, SI-YO SMB10; 13, No. 17.

These observations demonstrate the specificity of the primer pairs for each dengue virus serotype among the flaviviruses.

Reactivity of the primers to various isolates in Southeast Asia. We investigated the specificity of the primer pairs to dengue virus strains isolated from patients' sera in Southeast Asia. Figure 4 clearly shows the serotype specificity of the four primer pairs for the dengue type 2, 3, and 4 virus strains in Southeast Asia examined thus far.

DISCUSSION

The four sets of primers we selected each showed type-specific reactivity with sufficient sensitivity to the isolates collected in Thailand, Indonesia, and the Philippines as well as to prototype viruses, indicating that the sequences of each primer were well conserved in each dengue virus serotype isolate in Southeast Asia and highly specific to the respective serotype.

The C6/36 cells (10) can be used for dengue virus isolation from patients' sera and from field-caught mosquito homogenates as an improved virus isolation method (11, 19). We examined C6/36 cell culture fluids inoculated with patients' sera by using our rapid RT-PCR method and confirmed that the system worked very well on these primary infected cell culture fluids (data not shown). Since all procedures of rapid RT-PCR were completed within 2 h and the results of the serotypes were consistent with those determined by immunological examination (immunoperoxidase staining of the infected cells by using dengue virus type-specific monoclonal antibodies), in which 16 samples were in agreement (unpublished data), the method was shown to be useful for clinical and field specimens.

Since the number of cases of dengue virus infection is increasing (6), the disease is a serious health problem in tropical areas. It is important, therefore, to have a reliable method to prove the virus infection and to identify the serotype for clinical diagnosis and epidemiological study. Laboratory diagnosis of primary dengue virus infection can be performed by immunoglobulin M-capture enzyme-linked ...

virus, because of the "original antigenic sin" phenomenon (7). Needless to say, serodiagnosis can be made after the rise in antibody.

Thus, the most conventional and reliable method to determine the virus serotype is virus isolation from the patient's serum followed by immunological examination, using monoclonal antibodies (8, 9). If the method we describe is applied directly to dengue virus patients' sera, the diagnosis and virus serotype identification can be done at the same time with a single-tube reaction. We are planning the clinical application of this method in an epidemic area.

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Rapid Detection of Virus Genome From Imported Dengue Fever and Dengue Hemorrhagic Fever Patients by Direct Polymerase Chain Reaction

Kouichi Morita, Tatsuo Maemoto, Shoji Honda, Kenji Onishi, Misako Murata, Mariko Tanaka, and Akira Igarashi

Department of Virology, Institute of Tropical Medicine, Nagasaki University, Nagasaki (K.M., M.T., A.I.), Department of Pediatrics, Asahi-chuo Hospital, Chiba (T.M., S.H.), and Department of Communicable Disease, Bokuto Metropolitan Hospital, Tokyo (K.O., M.M.), Japan

Serum specimens from patients with imported dengue fever and dengue hemorrhagic fever were directly subjected to reverse transcription and polymerase chain reaction (RT-PCR) without any RNA purification. The amplified virus genome was detected within 3 hours. The results of PCR corresponded closely to the results of virus isolation using cultured mosquito cells, suggesting that direct RT-PCR procedure greatly facilitates rapid diagnosis of dengue infection.

KEY WORDS: virus diagnosis, direct RT-PCR, flavivirus

INTRODUCTION

Dengue/dengue hemorrhagic fever (DHF) caused by four serotypes of dengue virus is a significant health problem in tropical countries [Halstead, 1980, 1990]. The World Health Organization (WHO) estimates that 30–60 million cases occur every year around the world [WHO, 1990], including imported dengue cases found among travelers returning from tropical countries reported in nonepidemic countries such as the United States [CDC, 1991; Malison and Waterman, 1983; Morens et al., 1987; Scully et al., 1989], Japan [Okuno et al., 1989], New Zealand [Mills and Jones, 1991], South Africa [Blackburn and Rawat, 1987], and elsewhere.

Classical dengue is generally characterized by typical clinical manifestations and a benign prognosis. On the other hand, DHF often occurs among children and [illegible] prevent the disease [illegible] reversible shock [illegible] are essential for proper clinical treatment of DHF.

The polymerase chain reaction (PCR) has been utilized for the detection of dengue virus genome by several groups [Deubel et al., 1990; Eldadah et al., 1991; Henchal et al., 1991; Laille et al., 1991]. We have also established a rapid PCR method by which identification and serotyping of dengue virus can be done at the same time in a single tube reaction using dengue virus infected fluids [Morita et al., 1991]. Because dengue infection is an acute viral disease, a speedy diagnosis contributes enormously to the results of clinical treatment for affected patients.

We describe the modification of a rapid PCR method and its application to clinical specimens of imported dengue fever and DHF patients without RNA purification thereby achieving better clinical results.

PATIENTS AND METHODS

Patients

Patient 1 is a 2-year-old Japanese boy who had visited Bangkok, Thailand, and developed high fever and rash 3 days (November 18, 1991) after returning home. While in the hospital the patient had hepatomegaly, edema of the extremities, ascites fluid, and decrease of platelets, but the tourniquet test was negative.

Patient 2 is a 38-year-old Japanese man who developed a high fever during a trip to the Philippines and returned to Tokyo on the fourth day after onset (January 20, 1992). While in hospital he had petechiae, decrease of platelets and hemoconcentration, and the disease was diagnosed as grade II DHF.

Patient 3 is a 34-year-old Japanese nurse who had never been overseas but who suffered an accidental needle puncture while taking care of patient 2. She was hospitalized for high fever on the fifth day (January 25, 1992) after the accident but did not have any other remarkable complications.

Elevation of anti-dengue antibodies was confirmed in all three patients by the hemagglutination inhibition (HI) test and IgM capture enzyme-linked immunosor-

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Address reprint requests to Kouichi Morita, M.D., Ph.D., Department of Virology, Institute of Tropical Medicine, Nagasaki University, 12-4, Sakamoto Nagasaki, Japan 852.

TABLE I. Nucleotide Sequences of the Dengue Primers

Code[a]	Sequence	Target size (bps)[b]
D1S	GGACTGCGTATGGAGTTTTG	490
D1C	ATGGGTTGTGGCCTAATCAT	
D2S	GTTCCTCTGCAAACACTCCA	230
D2C	GTGTTATTTTGATTTCCTTG	
D3S	GTGCTTACACAGCCCTATTT	320
D3C	TCCATTCTCCCAAGCGCCTG	
D4S	CCATTATGGCTGTGTTGTTT	398
D4C	CTTCATCCTGCTTCACTTCT	

[a]D1,2,3,4: dengue type 1,2,3,4; S, sense primer; C, complementary primer.
[b]bps, base pairs.

Virus Isolation

Serum specimens from the patients were inoculated on *Aedes albopictus* cell clone C6/36 as described previously [Igarashi et al., 1982; Tesh, 1979]. The culture fluids of the inoculated cells were harvested on the fourth day after the inoculation and transferred to new C6/36 cells. On the third day of the second passage, the cells were harvested and stained by indirect immunoperoxidase staining using anti-dengue type-specific monoclonal antibody. The infected fluids were then examined by rapid reverse transcription (RT)-PCR as described previously [Morita et al., 1991].

Serological Examination

The HI test and IgM capture ELISA were carried out as described by Clarke and Casals [1958] and Bundo and Igarashi [1985], respectively.

Primers

All primers were synthesized using an Applied Biosystems DNA synthesizer (Model 392). The purity of the primers was confirmed by ion-exchange gel chromatography (Gen-pack, Waters). When more than 1% of incomplete length of oligomer was observed, the primer was discarded and the sequence was synthesized anew. The primer sequences used to detect each serotype of dengue virus and their target size are listed in Table I.

RT-PCR

Rapid RT-PCR was carried out as described previously [Morita et al., 1991], with some medification. In short, 5 μl of serum specimen was mixed with an equal volume of 1% Nonident-P 40 in phosphate-buffered saline [PBS(−)] with 100 units of RNasein (TAKARA Co., Japan) followed by 1 min incubation at room temperature. Ninety microliters of RT-PCR mix (100 pmol of sense and reverse primers, 0.2 mM dNTP, 10 mM Tris [pH 8.9], 1.5 mM $MgCl_2$, 80 mM KCl, 0.5 mg/ml bovine serum albumin [BSA], 0.1% sodium cholate, 0.1% Tri- [illegible] reverse transcriptase (Life Science [illegible] polymerase [illegible] Japan)) was added to the above sample. The tubes were set in a thermal programmer, oil bath type PCR machine (IWAKI Co. Tokyo, Japan), and subjected to programmed incubation: 5 min at 53 °C for RT once, followed by 40 PCR cycle (92 °C for 1 min, 53 °C for 1 min, 72°C for 1 min each). The entire procedure is shown as a schema in Figure 1. Four independent reaction tubes containing each dengue type-specific primer pair were run for each specimen.

After PCR amplification, 5 μl of PCR product was mixed with 1 μl of dye solution (1% sodium dodecyl sulfate [SDS], saturated bromophenol blue and xylene cyanol) and subjected to agarose gel electrophoresis. Amplified DNA fragments were visualized by ethidium bromide staining.

Southern Blot Hybridization

Amplified DNA band was denatured with alkali and Southern transferred onto nylon membrane. Oligonucleotide of the internal sequence of the target DNA, i.e., 5′-GGA,TTC,TGC,TGA,CAT,GGC,TAG,GAT,TAA,A-3′ (2286–2313 nt) for dengue 1, was labeled with horse radish peroxidase using the ECL 5′-thio oligo labeling system (Amersham) and hybridization and detection were performed according to the manufacturer's manual.

RESULTS

We applied this rapid RT-PCR procedure, which was shown to facilitate simultaneous identification and serotyping of dengue viruses using virus infected fluid [Morita et al., 1991], to serum specimens from three imported dengue patients.

At the beginning of the procedure, 5 μl of each serum was incubated with an equal volume of 1% Nonident P-40 and 100 units of RNase inhibitor in PBS. This treatment was necessary to disrupt virus particles and thus ensure that viral RNA could be a template for RT. The treatment has been effective on many other flaviviruses such as Japanese encephalitis virus, yellow fever virus, West Nile virus, Murray Valley encephalitis virus, and Saint Louis encephalitis virus for direct RT-PCR (data not shown). RNase inhibitor was also an indispensable reagent in this step of the direct RT-PCR because of the rich contamination of ribonuclease in human sera.

Figure 2 shows successful gene amplification using the method from the two dengue fever and one DHF patients described in the Patients and Methods section. The sera on days 3 and 5 after onset from these three patients did not possess any anti-dengue antibody. The serotype of the causative dengue virus was considered to be type 1 (D1) because of the target size in all three cases. Dengue viruses were also isolated from all three specimens and the serotype was confirmed using D1-specific monoclonal antibody. All procedures, from serum specimen processing to identification of virus serotype on agarose gel electrophoresis, were completed [illegible] PCR, virus isolation, and serological examination [illegible]

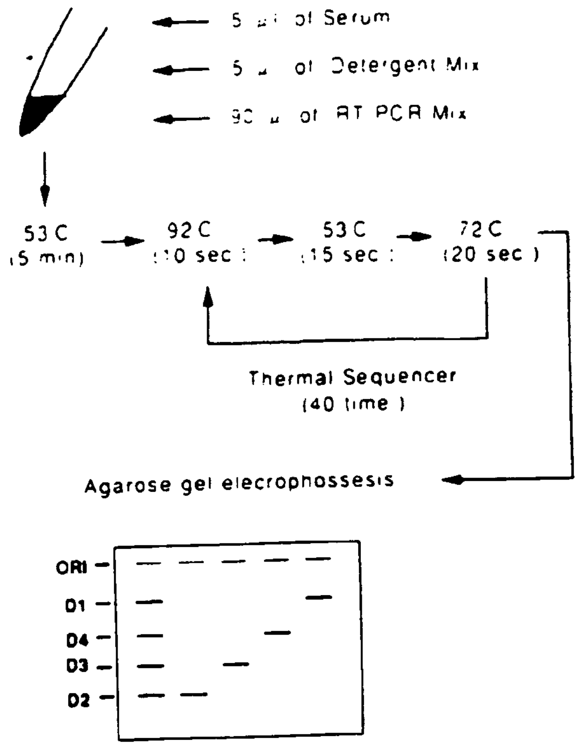


Fig. 1. Schema of direct rapid RT-PCR procedure.

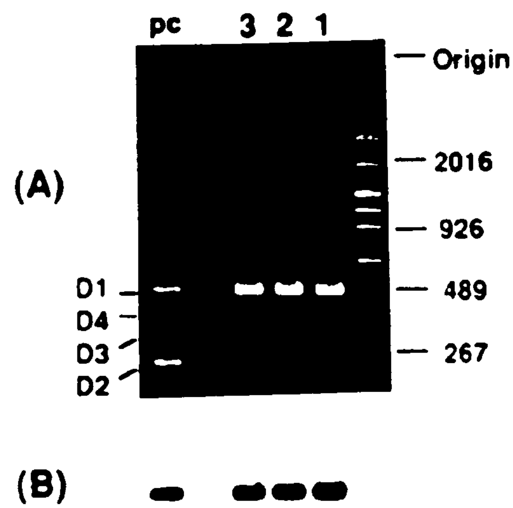
ble II). Virus genome was detected in the specimens from days 3 and 5 (Fig. 3) and the results were compatible with those of virus isolation. After day 8, the virus was no longer detected by PCR or virus isolation and anti-dengue IgM antibody was confirmed on days 8, 10, and 23.

This rapid method was also carried out using another D1-specific primer pair, D1(+) and D1(−), described by Deubel et al. [1990], and a positive result was obtained with the same convenience, suggesting that our procedure is useful for other primer sequences of flaviviruses (data not shown).

The efficiency of the one-step PCR described was compared with normal PCR performed after RNA extraction and ethanol precipitation (Fig. 4). The sensitivity of direct RT-PCR was one log lower compared with normal method where extracted RNA was used as templates.

DISCUSSION

... Burke ... Chungue ... Igarashi ... et al., 1989; Lam et al., 1987] and virus isolation [Igarashi et al., 1982; Lam et al., 1986; Rosen, 1981; Rosen and Gubler, 1974; Tesh, 1979]. However, virus isolation takes at least 1 week and serological examination can



... products ... Methods ... dengue type 1-4 ... Patient 1 on day ..., patient 2 on day ... patient 3 on day 3, respectively. Positive control was prepared by mixing four individual RT-PCR products from virus infected fluids. B: Southern hybridization of the above products with D1-specific oligonucleotide probe as described in the Patients and Methods section.

TABLE II. Results of Virus Isolation, Serological Examination and Polymerase Chain Reaction of Patient 1

Days after onset	PCR	Virus isolation	IgM capture ELISA	HI D1[a]	HI D2	HI D3	HI D4
3	+	+	—	<20[b]	<20	<20	<20
5	+	+	—	<20	<20	<20	<20
8	—	—	+	<20	<20	<20	<20
10	—	—	+	<20	<20	<20	<20
23	—	—	+	80	<20	20	160

[a]D1,2,3,4, dengue type 1,2,3,4.
[b]End point titers of HI test.

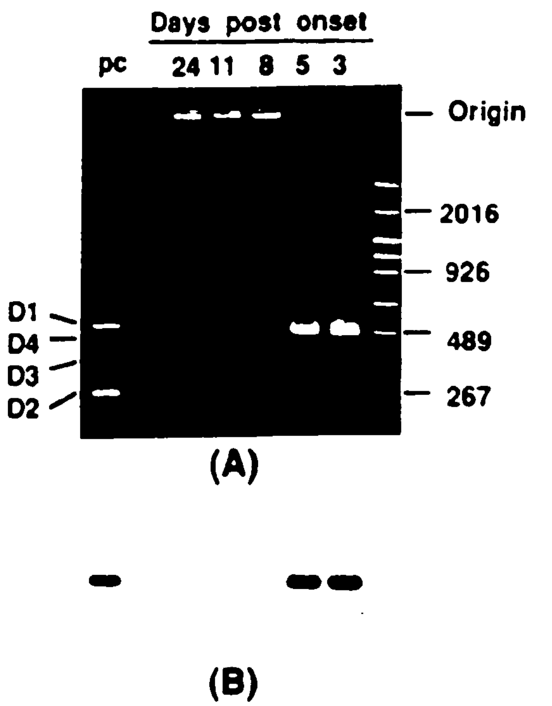


Fig. 3. Detection of D1 genome from the peripheral blood of patient 1 using D1-specific primer pairs. A: Serum specimens, collected from the patient sequentially, were subjected to direct RT-PCR procedure as described in the Patients and Methods section. The results using dengue 2,3,4-specific primer pairs were all negative (data not shown). Further confirmation of amplified DNA band by hybridizing of D1-specific oligonucleotide probe is shown B. D1, dengue 1; D2, dengue 2; D3, dengue 3; D4, dengue 4, positive control.

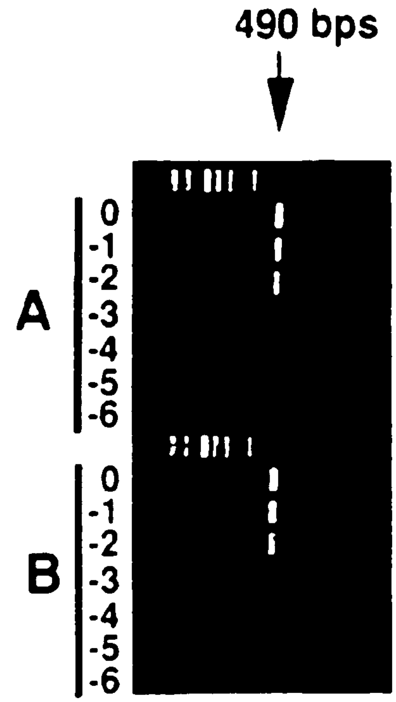


Fig. 4. Comparison of sensitivity between direct RT-PCR and normal RT-PCR after RNA extraction. A: Positive serum from patient 1 was diluted with normal human serum by ten fold dilution manner and the RNA was extracted by conventional phenol chloroform extraction. The extracted RNAs were subjected to RT-PCR as described in the Patients and Methods section. B: Diluted serum as described in (A) was subjected to direct RT-PCR procedure. The numbers indicate exponent of 10 in serum dilution.

be carried out only after the rise in antibody. These diagnostic methods are therefore of little use for clinical management.

Several groups of dengue investigators have already agreed on the usefulness of PCR as a rapid diagnostic method for dengue infection [Deubel et al., 1990; El- [illegible] tracted by conventional methods followed by cold ethanol precipitation and where cDNA was synthesized independently before PCR amplification [Deubel et al., 1990; Henchal et al., 1991; Laille et al., 1991]. These procedures still require much time and labor, and of course increase the chance of contamination.

We achieved a 3-hour diagnosis by template preparation with nonionic detergent and modification of the RT-PCR procedure. This procedure therefore satisfies [illegible] requirement of rapidity for practical diagnosis of [illegible] dengue cases reported clearly demonstrate [illegible] ness of direct RT-PCR before the appearance of [illegible] dengue antibodies.

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Rapid Detection and Typing of Dengue Viruses from Clinical Samples by Using Reverse Transcriptase-Polymerase Chain Reaction

ROBERT S. LANCIOTTI,* CHARLES H. CALISHER, DUANE J. GUBLER, GWONG-JEN CHANG, AND A. VANCE VORNDAM†

Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control, P.O. Box 2087, Fort Collins, Colorado 80522

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We report on the development and application of a rapid assay for detecting and typing dengue viruses. Oligonucleotide consensus primers were designed to anneal to any of the four dengue virus types and amplify a 511-bp product in a reverse transcriptase-polymerase chain reaction (PCR). First, we produced a cDNA copy of a portion of the viral genome in a reverse transcriptase reaction in the presence of primer D2 and then carried out a standard PCR (35 cycles of heat denaturation, annealing, and primer extension) with the addition of primer D1. The resulting double-stranded DNA product of the RT-PCR was typed by two methods: dot blot hybridization of the 511-bp amplified product to dengue virus type-specific probes or a second round of PCR amplification (nested PCR) with type-specific primers, yielding DNA products the unique sizes of which were diagnostic for each dengue virus serotype. The accumulated data demonstrated that dengue viruses can be accurately detected and typed from viremic human serum samples.

Dengue viruses (family *Flaviviridae*, genus *Flavivirus*) occur as four antigenically distinct serotypes. Infection with any of them generally leads to a mild, self-limiting febrile illness (dengue fever). However, a more severe form of the disease, involving vascular and hemostatic abnormalities (dengue hemorrhagic fever-dengue shock syndrome [DHF-DSS]), is responsible for a high mortality rate, primarily among children. Indeed, DHF-DSS is a leading cause of hospitalization and death among children in Southeast Asia, where more than one million cases were recorded between 1987 and 1989 (8). Over 30,000 deaths due to DHF-DSS in children have been reported worldwide since 1950 (7). Millions of human dengue infections occur each year, and over two billion people are at risk of infection.

These viruses are transmitted between humans primarily by *Aedes aegypti* and *Aedes albopictus* mosquitoes and are endemic in most areas in which the vectors occur (5). In dengue-endemic areas, dengue infections are recorded annually, with nonimmune children being the principal susceptible hosts. In addition, epidemics occur when a vector is introduced into previously dengue-free areas (8). The viruses replicate in cells of the macrophage-mononuclear cell lineage, and the severity of disease appears to be correlated with the ability of the viruses to infect these cells (7). Infection with one of the serotypes stimulates the production of neutralizing antibodies directed primarily against the envelope protein, conferring lifelong immunity to the serotype. The existence of waning neutralizing antibodies to one serotype may promote the enhancement of infection upon subsequent infection with another serotype. In this antibody-dependent enhancement model, severe disease is postulated to be the result of heterologous, nonneutralizing antibodies facilitating virus infection of mononuclear cells. Alternatively, it has been postulated that there exist viral and/or other host factors which may be primary risk factors in the production of more severe disease (5, 7).

Whether severe pathogenesis is caused by antibody-dependent enhancement or by some other mechanism, tools for rapid and specific laboratory diagnosis, including virus typing, are needed. Such diagnosis is necessary so that appropriate prevention, treatment, and control measures can be initiated and accurate epidemiologic data can be maintained. That one of the four dengue virus serotypes is responsible for a particular infection can be serologically deduced by traditional assays, including serum dilution-plaque reduction neutralization, complement fixation, or hemagglutination inhibition. The infecting serotype is inferred by measuring a fourfold or greater rise or fall in antibodies to the particular serotype. In practice, specific diagnosis often is not possible because of the extensive cross-reactivity of antibodies to flaviviruses, particularly between dengue viruses (10). Paired serum samples are needed; this requirement causes a delay in diagnosis, and results are rarely clear-cut.

Virus isolation from patient serum collected in the acute phase of illness or from arthropod vectors can be accomplished with cell cultures or mosquitoes. Currently, the most sensitive method of virus detection is inoculation of adult *A. aegypti* or *Toxorhynchites* species mosquitoes and fluorescent-antibody staining of mosquito brain tissues with dengue virus type-specific monoclonal antibodies (6). However, virus isolation takes from days to weeks and is not always successful because of small amounts of viable virus in the inocula, virus-antibody complexes, and inappropriate handling of samples. A clear need exists for an assay that can be performed rapidly and that is sufficiently sensitive and specific to be clinically and epidemiologically useful.

The development of the polymerase chain reaction (PCR)

TA[illegible]E 1. Oligonucleotide primers used to amplify an[illegible]engue viruses

Primer	Sequence	Genome position[a]	Size, in bp, of amplified DNA product (primers)[b]
D1	5'-TCAATATGCTGAAACGCGCGAGAAACCG-3'	134–161	511
D2	5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'	616–644	511
TS1	5'-CGTCTCAGTGATCCGGGGG-3'	568–586	482 (D1 and TS1)
TS2	5'-CGCCACAAGGGCCATGAACAG-3'	232–252	119 (D1 and TS2)
TS3	5'-TAACATCATCATGAGACAGAGC-3'	400–421	290 (D1 and TS3)
TS4	5'-CTCTGTTGTCTTAAACAAGAGA-3'	506–527	392 (D1 and TS4)

[a] The genome positions of D1 and D2 are given according to the dengue type virus 2 published sequence (2), and the map positions of the dengue virus type-specific primers (TS1, TS2, TS3, and TS4) are given according to their respective published sequences (2, 9, 12, 15).

[b] The size of the amplified product obtained with each of the type-specific primers (TS1 to TS4) was determined from the priming position of primer D1 within each respective genome. The priming position for D1 in each dengue virus genome was as follows: type 1, 105; type 2, 134; type 3, 132; and type 4, 136.

that would detect and correctly type dengue viruses in serum samples from humans with dengue fever or in mosquitoes infected with dengue viruses. The data presented in this paper demonstrate that this assay is sufficiently rapid and accurate to allow reliable case diagnoses and to be useful in epidemiologic studies.

MATERIALS AND METHODS

Virus strains. Virus seeds were obtained from the collection at the Division of Vector-Borne Infectious Diseases, Centers for Disease Control, Fort Collins, Colo. Prototype dengue virus strains (dengue virus types 1 [Hawaii], 2 [New Guinea C], 3 [H-87], and 4 [H-241]) were titrated in Vero cells by a standard plaque assay.

RNA extraction. Viral RNA was isolated by a modified form of the procedure described by Chomczynski and Sacchi (1). In brief, human serum samples or supernatant fluid from virus-infected cells was mixed with an equal volume of guanidine isothiocyanate lysis buffer: 8 M guanidine isothiocyanate, 50 mM sodium citrate, 100 mM 2-mercaptoethanol, 1% Sarkosyl, and 1 μg of yeast tRNA per ml. For RNA extraction from infected cells, we used a half concentration of lysis buffer. The solution was sequentially mixed with the following (all added in relation to the final volume of sample plus lysis buffer): a 1/10 volume of 2 M sodium acetate (pH 4), an equal volume of water-equilibrated phenol, and a 2/10 volume of chloroform. The mixture was centrifuged at 16,000 × *g* for 15 min, and the aqueous phase was removed and combined with an equal volume of isopropanol to precipitate the RNA. After centrifugation, the resulting RNA pellet was washed with 75% ethanol and dissolved in water. Control RNA used in sensitivity studies was quantitated by spectrophotometric analysis at 260 nm, and the concentration was calculated as follows: one unit of optical density = 40 μg/ml.

Selection and synthesis of oligonucleotide primers. Dengue virus consensus primers D1 and D2 were designed from available published sequences with the aid of a sequence analysis computer program (2, 9, 12, 15). The following criteria were used in designing the primers: (i) maximum homology to the four serotypes, (ii) high melting temperature, and (iii) nonhomology to other regions of dengue virus genomes. Primers D1 and D2 fulfilled these criteria and are shown in Table 1, along with their genome positions and product sizes when used in enzymatic amplifications. The type-specific primers shown in Table 1 (TS1, TS2, TS3, and

sizer and standard phosphoramidite chemistry and purified on a NENSORB (DuPont NEN, Boston, Mass.) column.

Amplification of dengue virus RNA. Target viral RNA was converted to a DNA copy (cDNA) prior to enzymatic DNA amplification by use of reverse transcriptase (RT) and the dengue virus downstream consensus primer (D2), homologous to the genomic RNA of the four serotypes. Subsequent *Taq* polymerase amplification was performed on the resulting cDNA with the upstream dengue virus consensus primer (D1). All relevant aspects of the RT-PCR ($MgCl_2$, primers, RT, *Taq* polymerase, number of cycles, and annealing temperature) were initially optimized by use of quantitated purified dengue virus RNA to achieve a maximum level of sensitivity. Of particular interest was the observation that rav-2 recombinant RT (Amersham, Arlington Heights, Ill.) consistently yielded at least 10-fold more amplified product than did Moloney murine leukemia virus RT obtained from a number of manufacturers (data not shown). The amplification reaction was routinely performed by combining the reverse transcription of viral RNA and the subsequent *Taq* polymerase amplification in a single reaction vessel. This method consistently yielded an equal or a greater level of double-stranded DNA product as separate RT reactions and PCRs. Target RNA was amplified in 100-μl volumes containing the following components: 50 mM KCl, 10 mM Tris (pH 8.5), 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM each of the four deoxynucleotide triphosphates, 5 mM dithiothreitol, 50 pmol each of primers 1 and 2, 2.5 U of rav-2 RT, and 2.5 U of Amplitaq polymerase (Perkin Elmer, Norwalk, Conn.). The reactions were allowed to proceed in an Ericomp (San Diego, Calif.) thermocycler programmed to incubate for 1 h at 42°C and then to proceed with 35 cycles of denaturation (94°C, 30 s), primer annealing (55°C, 1 min), and primer extension (72°C, 2 min).

Dengue virus typing by dot blot filter hybridization of the amplified product. A 10-μl portion of the RT-PCR mixture was denatured in 0.3 M NaOH at 65°C for 30 min and then immobilized on four separate Duralon membranes (Stratagene, La Jolla, Calif.) by use of a 96-well vacuum manifold. The DNA was fixed to the membranes by UV irradiation for 15 s with a UV Stratalinker 2400 (Stratagene) and then stored until tested by hybridization. Oligonucleotides for type-specific hybridization were 3' end labeled with digoxigenin (DIG)-UTP (Boehringer Mannheim, Indianapolis, Ind.) by combining the oligonucleotide (10 μM) with DIG-UTP (100 μM) and 15 U of terminal deoxynucleotidyl transferase (Life T[illegible] [illegible]nologies Inc., Gaithersburg, Md.) in the buffer sup-

poration and hence less sensitive in hybridization reactions (data not shown). Each membrane was hybridized with one of the four dengue virus type-specific oligonucleotides in hybridization buffer (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1% blocking reagent [Boehringer Mannheim], 0.1% *N*-lauroylsarcosine, 0.02% sodium dodecyl sulfate [SDS]) containing 100 ng of labeled oligonucleotide per ml. Hybridization reactions were performed for 16 to 20 h at 42°C. Membranes were washed twice for 20 min each time in 2× SSC–0.2% SDS and twice in 0.2× SSC–0.2% SDS. The bound probes were detected by incubation with alkaline phosphatase-labeled antibody to DIG and Lumi-Phos (Boehringer Mannheim) in accordance with the manufacturer's protocol. Visualization of bound probes was accomplished by exposing Kodak XAR film to the membranes for 3 to 15 min.

Dengue virus typing by second-round amplification with type-specific primers (nested PCR). A second amplification reaction was initiated with 10 μl of diluted material (1:100 in sterile distilled water) from the initial amplification reaction. The reaction mixture contained all the components described for the initial amplification reaction with the following exceptions: primer D2 was replaced with the dengue virus type-specific primers TS1, TS2, TS3, and TS4, and dithiothreitol and RT were eliminated. The samples were subjected to 20 cycles of denaturation (94°C, 30 s), primer annealing (55°C, 1 min), and primer extension (72°C, 2 min). A 15-μl portion of the reaction product was electrophoresed on a 4% composite agarose gel (NuSieve 3:1; FMC BioProducts, Rockland, Maine) in 0.4 M Tris–0.05 M sodium acetate–0.01 M EDTA buffer. Because of the position of priming with each of the dengue virus type-specific primers (Table 1), the size of the resulting DNA band was characteristic for each dengue virus type.

Infection of mosquitoes and verification of infection. Insectary-maintained *A. aegypti* mosquitoes were infected by intrathoracic inoculation with undiluted human serum that had been shown in other assays to contain dengue type 2 virus. Mosquitoes were incubated at 30°C and 60 to 75% relative humidity. Pools consisting of five mosquitoes were removed daily, beginning 2 days after inoculation, and frozen for RT-PCR analysis. At 10 days after inoculation, dengue type 2 virus infection was verified by testing of a random sample of these mosquitoes by a direct immunofluorescence assay (DFA) of head-squash material with a conjugate prepared from high-titer human serum.

Detection of dengue viruses in mosquitoes by RNA capture prior to amplification. Amplification of RNA isolated from dengue virus-infected mosquitoes initially yielded negative results. Because the mosquitoes were known to be infected, as verified by the DFA, we postulated that an inhibitory component was present in the isolated RNA. To resolve this problem, we used a dengue virus RNA capture step prior to the RT-PCR. The D2 consensus primer was 3′ end labeled with biotin-14-dATP by use of terminal deoxynucleotidyl transferase as described above for DIG-UTP. The labeled oligonucleotide was immobilized on strepavidin-coated magnetic beads (Dynabeads; Dynal, Great Neck, N.Y.) by combining 100 μl of the bead suspension (binding capacity, 200 pmol of labeled oligonucleotide) with 200 pmol of the biotinylated oligonucleotide. After 10 min of incubation at room temperature, the beads were washed four times in bead wash buffer (0.2 M Tris [pH 7.5], 0.2 M NaCl) by use of a

[illegible]

5 min and slowly [illegible] to 42°C for 5 min to allow the RNA to anneal. The beads were washed twice with bead wash buffer and mixed with 10 μl of water, and the mixture was heated to 70°C to elute the RNA.

Detection and typing of dengue viruses from viremic human serum. Human serum samples were obtained from patients with clinically characterized and virologically confirmed dengue infection and were tested by the RT-PCR assay. These samples had previously been shown to contain dengue viruses by isolation in C6/36 *A. albopictus* cell cultures or by intrathoracic inoculation of mosquitoes and the DFA as described above. Dengue virus serotypes were determined by an indirect immunofluorescence assay (IFA) with dengue virus type-specific monoclonal antibodies (6). We tested samples obtained from persons with either classical dengue fever or DHF-DSS during several epidemics in Southeast Asia and Puerto Rico. The samples from Southeast Asia had been stored frozen at −70°C with occasional to multiple thawings over a 10- to 15-year period. The samples from Puerto Rico were from more recent cases (less than 1 year before our test) of dengue fever in Puerto Rico.

RESULTS

Specificity of the RT-PCR. RNA isolated from each of the four dengue virus reference strains was subjected to the RT-PCR assay. The correctly sized DNA product (511 bp) was obtained for each of the dengue viruses after amplification with consensus primers D1 and D2 (Fig. 1A). Each DNA product was correctly typed when assayed by either dot blot hybridization with the type-specific probes (Fig. 2) or a second round of amplification with the type-specific primers (Fig. 1B). The specificity was also verified by performing the nested PCR assay on 33 unique dengue virus isolates representative of most of the defined geographic topotypes (Table 2) (14). In addition, the RT-PCR assay was tested for specificity by attempting amplification reactions with purified RNA from five dengue virus-related flaviviruses (West Nile, Japanese encephalitis, St. Louis encephalitis, yellow fever, and Edge Hill). Viruses of the Japanese encephalitis complex (Japanese encephalitis, West Nile, and St. Louis encephalitis) were amplified in the first-round amplification reaction with consensus primers D1 and D2 to generate DNA products of 511 bp for West Nile and St. Louis encephalitis virus and 550 bp for Japanese encephalitis virus, in agreement with the published sequences (Fig. 1A). Yellow fever and Edge Hill viruses were not amplified with the consensus primers (Fig. 1A). A faint DNA band of approximately 150 bp was observed for Edge Hill but was likely due to nonspecific amplification, since products of this size were occasionally observed in other PCRs. The DNA product obtained after first-round amplification of Japanese encephalitis, West Nile, and St. Louis encephalitis viruses did not react with the dengue virus type-specific oligonucleotide probes in dot blot hybridization experiments (Fig. 2). In addition, no DNA products were obtained when these amplified DNAs were used as targets in the nested PCR amplification with the dengue virus type-specific oligonucleotide primers (Fig. 1B). First-round amplification of related flaviviruses did not alter the specificity of the assay, since the amplified DNA products generated did not react with the dengue virus type-specific probes or primers.

Sensitivity of the RT-PCR. For postamplification detection ... [illegible] the sensitivities of two methods were initially

[illegible]

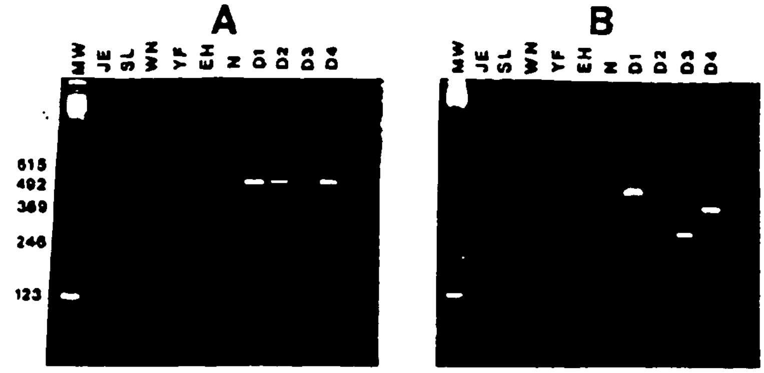
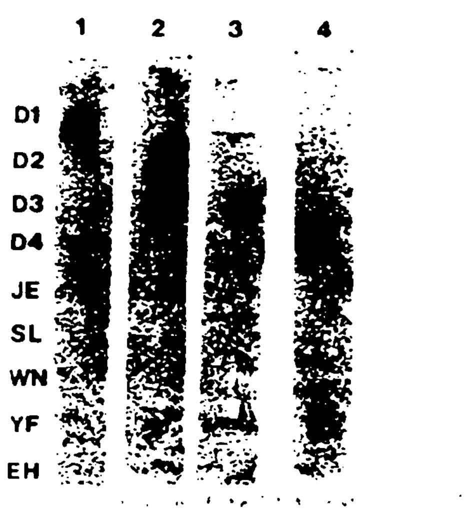


FIG. 1. Agarose gel analysis of the DNA product from RT-PCR of RNA samples isolated from dengue viruses and related flaviviruses. (A) After amplification with consensus primers D1 and D2. (B) After second-round amplification with type-specific primers TS1, TS2, TS3, and TS4. Molecular weight (MW) markers are shown on the left; DNA sizes are given in base pairs. Lanes show amplification of RNA from the following viruses: JE, Japanese encephalitis; SL, St. Louis encephalitis; WN, West Nile; YF, yellow fever; EH, Edge Hill; N, western equine encephalitis (negative control); D1, dengue type 1; D2, dengue type 2; D3, dengue type 3; and D4, dengue type 4.

hybridized with each of the four dengue virus type-specific probes labeled with DIG-UTP; initially ^{32}P-labeled probes were used, but these were later replaced with DIG-UTP probes of equal sensitivity. Using purified RNA as a standard, we consistently attained a sensitivity level of between 1,000 and 100,000 viral genome equivalents (Fig. 3). In the second protocol (nested), a small portion of the amplified product was subjected to an additional 20 cycles of amplification with the D1 consensus primer in combination with the four type-specific primers. Figure 4 displays the results of applying this nested PCR method with the same samples as those used in the hybridization analysis. Sensitivity attainable by this nested amplification method was greater; 100 viral genome equivalents were detected. The two protocols were also compared by testing 20 human viremic serum samples. The nested approach proved more sensitive by



[illegible] ... to the left are as defined in the legend ... [illegible] Membranes were hybridized with probes specific for dengue viruses of types 1 (lane 1), 2 (lane 2), 3 (lane 3), and 4 (lane 4) [illegible] probes were labeled with DIG-UTP and detected with Lumi-Phos

TABLE 2. Comparison of RT-PCR and serological typing of geographically and temporally distinct dengue viruses

Strain	Location	Yr isolated	Serotype determined by both methods
16007	Thailand	1964	1
1041	Indonesia	1976	1
30893	Malaysia	1981	1
162.AP2	Philippines	1984	1
11651	Puerto Rico	1986	1
GML100063	Guatemala	1989	1
INS353117	Columbia*	1990	1
INS353178	Colombia*	1990	1
88970	Venezuela	1990	1
TC16681/64	Thailand	1964	2
489	Puerto Rico	1977	2
285	Indonesia	1978	2
042.AP4/2207	Philippines	1983	2
D85-044	Thailand	1985	2
1715	Dominican Republic	1986	2
88967	Venezuela	1990	2
CH53489D731	Thailand	1973	3
1300	Malaysia	1974	3
1340	Puerto Rico	1977	3
1178	Indonesia	1977	3
1280	Indonesia	1978	3
D80273	Thailand	1980	3
26237	Malaysia	1980	3
168.AP2	Philippines	1983	3
D84315	Thailand	1984	3
1594	Sri Lanka	1985	3
D86013	Thailand	1986	3
1053	Indonesia	1976	4
1132	Indonesia	1977	4

[illegible] South America

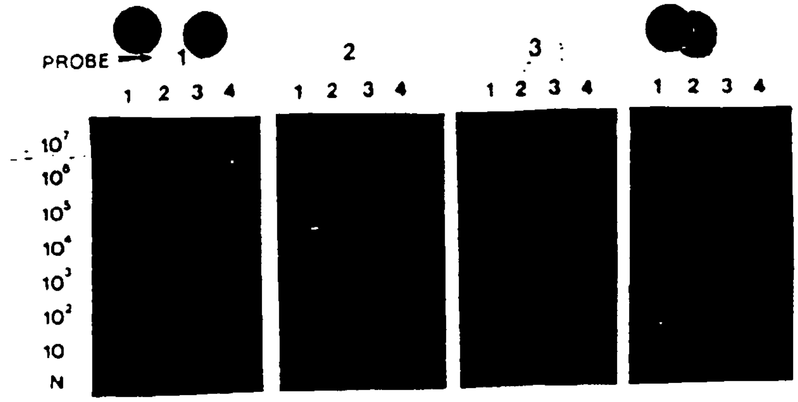


FIG. 3. Dot blot hybridization of the DNA product obtained after one round of RT-PCR amplification of quantitated dengue virus RNAs with consensus primers D1 and D2. The membranes contained identical samples in the same configurations. Lanes 1 to 4 show dengue viruses of types 1 to 4, respectively. Membranes were hybridized with probes specific for dengue viruses of types 1 (panel 1), 2 (panel 2), 3 (panel 3), and 4 (panel 4). All probes were labeled with DIG-UTP and detected with Lumi-Phos. The number of initial RNA molecules assayed is shown on the left.

correctly identifying five viremic serum samples that were found negative by the dot blot hybridization method (Table 3). The nested PCR method was used exclusively throughout the remainder of this study.

Detection and typing of dengue type 2 virus in infected mosquitoes. Figure 5 displays the results of testing dengue type 2 virus-infected *A. aegypti* mosquitoes. As previously stated, the RNA isolated from these infected mosquitoes was originally found negative for dengue type 2 virus by the RT-PCR assay at all time points. However, when the RNA samples were captured on magnetic beads prior to RT-PCR amplification, they were amplified with consensus primers D1 and D2 and correctly typed by the nested PCR method. Samples were found positive for dengue type 2 virus starting at the earliest time point (day 2) and were positive throughout the remainder of the time points assayed (Fig. 5).

Detection and typing of dengue viruses in clinical samples. Ninety-three human viremic serum samples were tested by the RT-PCR assay. Table 4 summarizes the results comparing identification by the RT-PCR assay with identification by virus isolation in mosquitoes or cell cultures and subsequent typing by the IFA with type-specific monoclonal antibodies. In all but four instances, dengue viruses were correctly detected and typed by the RT-PCR assay, compared with virus isolation. One dengue type 1 virus sample and three samples containing dengue type 2 virus were not found positive by the RT-PCR method. Ten additional samples from Southeast Asia (data not included in Table 4) were originally dengue virus positive when isolated but negative when tested by the RT-PCR assay. Since the storage history of these samples may have reduced or eliminated virus titers, these samples were reinoculated into mosquitoes and assayed for viruses. Seven samples were negative, and three samples yielded questionable results (one or two fluorescent cells were observed in the DFA). These three samples were subsequently passed in C6/36 cells. After a suitable period of incubation, two tested positive for dengue type 2 virus and one tested positive for dengue type 1 virus.

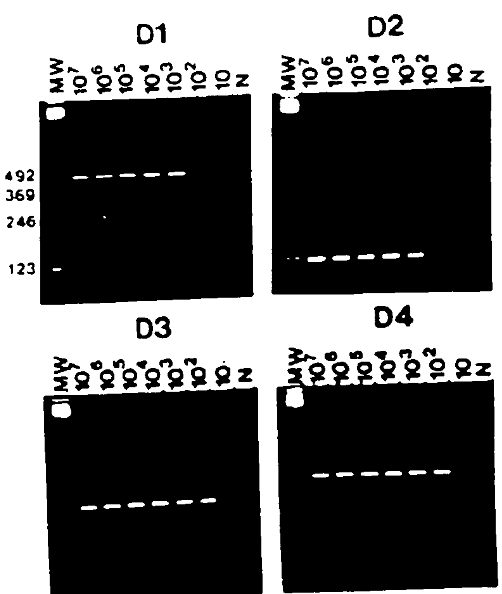


FIG. 4. Agarose gel analysis of the product from RT-PCR followed by second-round nested PCR of quantitated RNA samples

DISCUSSION

In this report, we describe the development of a rapid and specific assay for detecting and typing dengue viruses. The

TABLE 3. Comparison of dot blot hybridization and nested PCR for detection and typing of dengue viruses in human serum samples

Method	No. of viremic serum samples of the following dengue virus type:			
	1	2	3	4
[illegible]	2	6	[illegible]	[illegible]

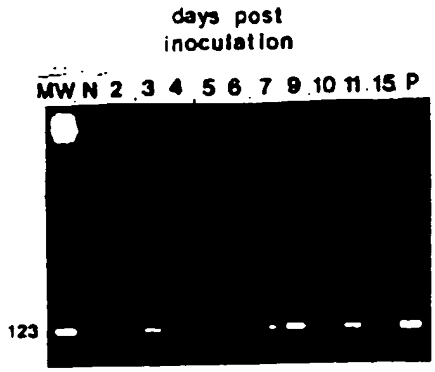


FIG. 5. Agarose gel analysis of the product from RT-PCR amplification of RNAs isolated from infected mosquitoes and captured on magnetic beads. Lanes: MW, molecular size markers (in base pairs); N, uninfected mosquitoes; 2 to 15, RNAs isolated from mosquitoes on the day postinfection shown above each lane; P, dengue type 2 virus positive control.

method relies on a combination of two steps: generation of a cDNA copy of the RNA genome by RT and subsequent *Taq* polymerase-mediated amplification of this cDNA. The two reactions are combined in a single reaction vessel, significantly reducing the assay time, lowering the risk of contamination problems, and facilitating the handling of large numbers of specimens. The use of primers homologous to conserved dengue virus RNA sequences ensures that all strains of dengue virus will be amplified in the first-round amplification reaction. The fact that viruses of the Japanese encephalitis serogroup were also amplified by the consensus primers confirms the broad reactivity of these primers. The use of type-specific primers for viruses of the Japanese encephalitis complex in similar two-round amplification assays would generate similar detection and typing tests for these viruses.

The specificity of our assay relies on the ability of the type-specific primers to recognize RNA sequences unique to each dengue virus type. This specificity was confirmed by testing 33 geographically unique virus isolates (characterized by RNA fingerprinting techniques [14]) as well as 93 previously identified viremic serum samples (Table 4). No cross-reactivity was detected between the type-specific primers and heterologous dengue virus types; only a single amplified product was obtained in each typing reaction. Typing of dengue viruses by the nested PCR method with a mixture of type-specific primers is superior to hybridization both in sensitivity and in ease of manipulation. Correct typing requires only electrophoresis of the amplified product on an agarose gel, whereas the hybridization method introduces a filter hybridization protocol requiring the labeling, purification, and standardization of probes. These probes, along with the hybridization protocol itself, are usually difficult to reproduce.

TABLE 4. Comparison of the RT-PCR assay and virus isolation for the identification and typing of dengue viruses from human serum

Method	No. of serum samples of the following dengue virus type:

The potential diagnostic usefulness of our assay is demonstrated by the analysis of human serum samples containing dengue virus. The assay demonstrates sensitivities of 94% with dengue type 1 virus, 93% with dengue type 2 virus, and 100% with dengue type 3 and 4 viruses, compared with virus isolation. The samples from Southeast Asia were originally titrated in mosquitoes and possessed virus titers ranging from 10^3 to 10^8 50% infective doses per ml of serum. A meaningful correlation between the original virus titers and the RT-PCR results was not possible because of the uncertain storage history of the samples. However, it is noteworthy that several of the samples which tested positive in the RT-PCR originally possessed virus titers as low as 10^3 50% infective doses per ml of serum. The four RT-PCR-negative samples that were found positive by virus isolation (false-negatives) may have been the result of the presence of fewer than 100 complete virus particles, the approximate sensitivity limit of the test. Another possibility is that these serum samples contained an inhibitor of the enzymatic amplification that copurified with the template RNA. A bead capture step could be used to eliminate this problem, as was done with RNA isolated from dengue virus-infected mosquitoes; however, insufficient sample volumes prevented execution of the bead capture step on these samples.

Although false-positive PCR results have been reported (13) in PCR-based assays, this problem was circumvented by routinely exercising numerous precautionary measures (physical separation of pre- and post-PCR manipulations, UV irradiation of reaction mixtures, and the use of positive-displacement pipettes) and including several samples without DNA to carefully monitor each assay.

Other reports in which PCR was used to identify dengue viruses have appeared (3, 4). Our assay possesses several differences which we believe make it more amenable to routine use in a diagnostic setting. First, the use of broadly reactive consensus primers for initial amplification ensures that all dengue virus isolates encountered in a diagnostic laboratory will be correctly identified. Second, the nested PCR method is both more sensitive and easier to standardize than either hybridization or restriction digestion for confirmation of the amplification product. Finally, RNA capture on magnetic beads prior to amplification allows circumvention of potential PCR inhibitors, which are likely to be encountered in the analysis of a large number of specimens.

The accuracy and speed of the RT-PCR assay make it an appealing test for the diagnosis of dengue and for epidemiologic surveillance. In our laboratory, we have been able to complete the RT-PCR assay, starting from RNA extraction and completing with agarose gel analysis, within 30 h. In diagnostic laboratories currently using traditional isolation or serological methods, this assay could be used to complement existing techniques or in some cases to replace them. In addition, the basic methodology of directly amplifying RNA into double-stranded DNA can be used to amplify larger regions of the genome for rapid sequence analysis, which is potentially useful for both epidemiologic analysis and evolutionary studies.

ACKNOWLEDGMENTS

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SENSITIVITY AND SPECIFICITY OF A UNIVERSAL PRIMER SET FOR THE RAPID DIAGNOSIS OF DENGUE VIRUS INFECTIONS BY POLYMERASE CHAIN REACTION AND NUCLEIC ACID HYBRIDIZATION

ERIK A. HENCHAL, STEPHANIE L. POLO, VANCE VORNDAM, CHANDHANA YAEMSIRI, BRUCE L. INNIS, AND CHARLES H. HOKE

Department of Virus Diseases, Walter Reed Army Institute of Research, Washington, DC; Centers for Disease Control, San Juan, Puerto Rico; Department of Virology, Armed Forces Research Institute of the Medical Sciences, APO San Francisco, California

Abstract. A set of sense and anti-sense oligomeric DNA primers, degenerate in the thir "wobble" base position of codons so as to match all known dengue virus sequences, wa evaluated as universal primers in a polymerase chain reaction (PCR) assay for the rapi diagnosis of dengue virus infections. Virus-specific complementary DNA (cDNA) wa prepared by reverse transcription (RT) of total RNA extracted from serum. Amplifie cDNA was identified by nucleic acid hybridization with four serotype-specific, oligomeri DNA probes. Using sera from patients admitted with dengue fever, RT/PCR followed by nucleic acid hybridization using radiolabeled probes was 68% sensitive (50/74; 95% confidence interval [CI] = 57–78%) and 100% specific. Chemiluminescent detection of hybridized products was 62% sensitive (26/42; 95% CI = 46–75%). Using specimens from which a virus isolate had been obtained, RT/PCR followed by nucleic acid hybridization with radiolabeled probes was 80% sensitive (40/50; 95% CI = 69–91%) and 100% specific. The results suggest that RT/PCR using degenerate primers is a sensitive and specific method for the detection of dengue viruses in clinical specimens.

Dengue, a major public health problem in tropical and sub-tropical regions, is the arthropod-borne viral disease of humans with the greatest morbidity and mortality.[1,2] Laboratory diagnosis of dengue currently depends upon detection of virus-specific antibodies in patient sera or isolation of infectious virus followed by serologic typing.[2] A four-fold or greater increase in antibody titer is diagnostic for a recent flavivirus infection using the hemagglutination-inhibition (HI) assay, the most widely used diagnostic test.[3] Enzyme-linked, immunosorbent assays (ELISA) for the detection of dengue virus-specific IgM were 10–78% sensitive for the laboratory diagnosis of dengue when admission sera were tested.[4–8] However, these assays can not be used to identify the serotype of the infecting dengue virus.

Extensive cross-reactions among the flaviviruses and the existence of four distinct dengue virus serotypes make etiologic identification by serologic methods difficult.[9–12] Identification of the infecting dengue virus serotype has depended upon isolation of virus in a sensitive host system. These methods result in the successful isola of virus and subsequent serotype identificat in 20–80% of specimens from serologically-c firmed dengue patients.[2] Virus isolation meth followed by serologic identification usually t two or more weeks, delaying laboratory diagnc until long after resolution of the patient's illn

The polymerase chain reaction (PCR) meth which allows for the multi-fold amplification viral nucleic acid, has been used to rapidly agnose viral diseases.[16–21] Recently, Deubel a others adapted the method for the detection dengue viruses using a mixture of four prim sets derived from sequences coding the N-te minus of the envelope protein. A PCR-amplifie serotype-specific complimentary DNA (cDN was cloned and used as a non-radioactive, n cleic acid hybridization probe in subsequent a says.[22]

Intratypic genetic variation among the dengu viruses, even in the same epidemic, is well es tablished.[16–28] The selection of PCR primers re quires careful evaluation, since the potential fo false negative results due to nucleotide-base mis

... cotide primers, degenerate in the third "wobble" base position of codons, in rapid and ...tive assays based upon PCR amplification.

MATERIALS AND METHODS

...

...he cultivation of dengue viruses in C6/36 (...s *albopictus*) cells and titration in LLC-MK2 (...nkey kidney) cells have been described pre...usly.[14] Dengue virus strains used as virus ...dards in this study included dengue-1 (Ha...), dengue-2 (Thailand, D80-100), dengue-3 (...), dengue-4 (Dominica, ...) Thailand, CH53489), and dengue-4 (Dominica, ...4669). The serotype identity of all dengue vi...ses was verified by indirect immunofluorescent ...says using serotype-specific monoclonal anti...dies as previously described.[15] Yellow fever (...D) and Kunjin viruses were maintained as ...w passage C6/36 cell culture stocks. Japanese ...cephalitis (Nakayama) and St. Louis enceph...itis viruses were maintained as high-passage ...ckling mouse brain stocks. Yellow fever virus was a gift of Dr. Robert Putnak (Walter Reed Army Institute of Research). Other non-dengue ...rus stocks were provided by Jack McCown (Walter Reed Army Institute of Research). Viral supernatants used for specificity testing con...ined at least 10^5 plaque-forming units/ml.

Patient specimens

Acute sera from 74 serologically-confirmed dengue fever patients and from seven patients with a non-dengue illness were obtained blind and under code from the Centers for Disease Control, San Juan, Puerto Rico and the Department of Virology, Armed Forces Research Institute of the Medical Sciences, Bangkok, Thailand. Dengue infections were diagnosed by the detection of anti-dengue IgM or increasing anti-dengue hemagglutination-inhibiting antibodies in paired sera.[3,4] Dengue viruses were isolated using C6/36 cells or by intrathoracic mosquito inoculation from 50 of 74 acute sera, and serotyped using reference monoclonal antibodies as previously described.[14,15,30,31] The mean infective dose $(MID)_{50}$, the reciprocal of the dilution infecting 50% of the mosquitoes inoculated, was determined for 22 specimens.[30] Normal human sera (flavivirus-naive) and a reference, virus-negative pooled human convalescent serum containing anti-flavivirus antibodies were included in some assays. The latter serum had a plaque-reduction neutralization titer > 1:160.[32]

Preparation of viral RNA

Viral RNA was prepared by guanidine isothiocyanate-phenol-chloroform extraction by a modification of the method described by Chomczynski and Sacchi.[33] One hundred microliters of serum or virus-containing fluid was mixed with 400 µl of solution D (4M guanidine isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol). Pooled human sera containing anti-dengue neutralizing antibodies was used as a diluent for stock virus in some experiments. Sodium acetate, pH 4.0, was added to a final concentration of 0.2 M, and the solution was extracted with an equal volume of water-saturated phenol and one-fifth volume of chloroform-isoamyl alcohol (49:1). The sample was mixed vigorously for 10 seconds and cooled on ice for 15 min. It was then centrifuged at 10,000 rpm in a Sorvall 33-34 rotor (Dupont, Wilmington, DE) for 20 min at 4°C. The top aqueous layer containing the viral RNA was transferred to a fresh tube and precipitated with an equal volume of ice cold isopropyl alcohol for one hr at −20°C. The precipitated RNA was collected by centrifugation at 10,000 rpm for 20 min at 4°C. The supernatant was aspirated from the tube, and the RNA was resuspended in 500 µl of 4 M lithium chloride. The RNA was collected by centrifugation as described. The pelleted RNA was resuspended in 150 µl of fresh solution D and 150 µl of ice-cold isopropyl alcohol. The RNA was reprecipitated for 1 hr at −20°C and collected by centrifugation as described. The pellet was washed with ice-cold 75% ethanol, collected by centrifugation as described, and dried using a Savant (Farmingdale, NY) speed vac concentrator.

Synthetic DNA oligomeric probes and primers

Reagent grade synthetic DNA oligomeric probes and primers (Table 1) were produced by Synthecell (Rockville, MD). Primer sequences bracketed a 419-nucleotide base sequence in the NS1 gene (Table 2), corresponding to bases 2981 to 3400 in the dengue-1 (Nauru Islands) genomic RNA sequence.[34] Identical deduced amino acid sequences occur at the primer annealing sites for

TABLE 1
Sequences of oligomeric DNA primers and probes for PCR amplification of dengue virus RNA

Oligomer*	Number	Sequence†
Primers		
AD3 (−)	—	CTGATTTCCAT(A, C, G, T)CC(A, G)TA
AD4 (+)	—	GA(C, T)ATGGG(A, C, G, T)TA(C, T)TGGATAGA
Probes		
D1 (+)	3135	GAGGACCAATATCTCAG
D2 (+)	3235	AAGCTTGAGATGGACTTT
D3 (+)	3150	GTCTAGCTGGTCCCATT
D4 (+)	3155	ATCATATGCGGGCCCTT

* Oligomers were either of genomic (+) or anti-genomic (−) sense. Numbers correspond to nucleotide base sequence. D1, dengue-1 specifi dengue-2 specific.[11] D3, dengue-3 specific.[16] D4, dengue-4 specific.[17]
† Sequences are presented in the 5' to 3' direction. Bases in parentheses are alternative bases at the sequence position shown.

dengue-2 (PR159/S1),[15] dengue-3 (H87),[16] and dengue-4 (Dominica/814669)[17] viruses. Primer sequences were degenerate in the third base position of codons, thus allowing detection of viruses with nucleotide sequence variations, but without amino acid sequence variations. Bases in parentheses (Table 1) are alternative bases at the nucleotide position shown. Synthetic DNA probe sequences were derived from serotype-specific base sequences within the PCR-amplified regions. Primer and probe sequences were designed to have three or more nucleotide mismatches with the sequences of non-dengue flaviviruses (unpublished data).

Reverse transcriptase-polymerase chain reaction assay (RT/PCR)

Virus-specific cDNA was amplified by RT/PCR using the universal dengue virus-specific, anti-sense oligonucleotide primer AD3 (Table 1). Dry RNA pellets were resuspended in 25 µl of sterile distilled water containing 25 units of RNAsin (human placental ribonuclease inhibitor; Promega, Madison, WI) or 0.1% diethyl pyrocarbonate (DEPC), heated at 68°C for 3 min, and placed on ice. Five µl of RNA was added to a 7.5 µl solution containing 11.8 pmoles of primer AD3 in sterile distilled water. The sample was heated at 68°C for 3 min, cooled on ice, and added to a reaction mixture containing 50 mM Tris, pH 8.3, 70 mM potassium chloride, 10 mM magnesium chloride, 10 mM dithiothreitol, 275 µM deoxynucleotide triphosphates, 25 units RNAsin, and 10 units of reverse transcriptase in

cDNA was performed by adjusting the mix to 10 mM Tris-HCl, pH 8.3, 50 mM potass chloride, 1.5 mM magnesium chloride, 20 ammonium sulfate, 200 µM deoxynucleotid phosphates, 0.3 µM primer AD3, 0.3 µM pri AD4, and 0.025 units/µl of Amplitaq® D polymerase in a total volume of 100 µl. The action mixture was heated at 94°C for 1 min, then cycled 29 times at 45°C (1 min), 72°C min), and 94°C (1 min). Samples were coole 4°C after a final incubation for 5 min at 72 Sample (15 µl) aliquots were electrophoresed 1.2% agarose gels in tris-borate-saline buffer stained with ethidium bromide using stand methods.[18] Stained gels were photographed o light box using Polaroid type 667 film. Some g were Southern blotted to Hybond-N membr and hybridized with mixtures of dengue seroty specific synthetic DNA probes.[18]

Preparation of labeled oligomeric probes

Oligomeric DNA probes specific for each the dengue viruses (Table 1) were radiolabel or modified for non-radioactive detection. R diolabeled probes were 5' end-labeled with using T4 DNA kinase, and partially-purified u ing Sephadex G-25 column chromatography, a cording to standard methods.[18] Radiolabele synthetic probes were diluted to 10⁶ cpm/ml i hybridization buffer before use. Non-radioacti probes were prepared with a terminal deoxyn ucleotide transferase assay in a reaction mixtur containing each of the four deoxynucleotide tri phosphates at a final concentration of 0.1 mM

...phosphate Boehringer Mannheim, ... at a final concentration of 0.03.

DEN 1	GACATGCGCT	ACTGGATAGA*	AACTGAAAAC	AACGAGACTT	GGAACTTGCC
DEN 2	. T . . . T .	T	. C C . CTC	T . T . A	. . . A A A
DEN 3	. . . . . . C .	. T	. . . CC	. . T GA . G .	. . . . C A A
DEN 4	. . . . T .	. T	G . . CTC . . . A	. . . C	. . C . . A A . A
AAGAGCCTCC	TTCATAGAAG	TTAAGACATG	CATCTGGCCA	AAATCCCACA	CTCTATGGAG
G . A . . . T . . T	. . . . . T	C . . A . GT . .	. CA	. . G . . A . . .	. . . .
. A . . . A . . .	C . . . . . . G .	. G . . A . . C . .	. . CA	. . . . . A	. . . . T . . . . .
G . A . . . A . T	C . T . . T	. G . . A	TC . G C	. . GA	. A . . G . . . . .
CAATGGAGTC	CTGGAAAGTG	AGATGATAAT	CCCAAAGATA	TATGGAGGAC	CAATATCTCA
T . . . . G	. . A . . . . . C .	. . . . . G	T . . . . AT	. TC . CT	. . G . G . . A . .
. . . . . . T . . G	. . A . . G	. C . . . . . T . .	. . . . GT	CTA . CT . . T .	. C . . T . . G .
. . . . G	 CC	. . . . . C . C . .	T . . . . . ATC .	. . . . . CG . . C .	. TT . T . . A .
GCACAACTAC	AGACCAGGAT	ATTTCACACA	AACAGCAGGG	CCGTGGCACT	TGGGCAAGTT
A . . T . . TA . .	. . . . C .	. . CA	. . . . A	. . T . . . . . TC	. A . . . . . . C .
A . . . . . . C . .	. . G . . C . . G .	. CCA . . . C . .	. . . G . . . . . A	. . C	. A . . A . . A . .
. . . . T . . .	C . C . A . . . C .	. . GC . . . G . .	. . . C . TG . . C	. . A	. A . . . . . A . .
AGAACTAGAT	TTTGATTTAT	GTGAAGGTAC	CACTGTTGTT	GTGGATGAAC	ATTGTGGAAA
T . . GA . G . . C	. . . . C .	. C . . . . . G . .	T . . A . . G . . G	. . AACC . . GG	C . . . .
G . . G . . G . . C	. CA . C . AT .	. . . . A . .	A . . A C	A . CTCA . . . A	. C . . . . . G . C
. . GA C	. . GAGA . .	. CCCC . . A . .	A . CG . . CACA	A . TC . G . . GG	. . . . . . ACC .
TCGAGGACCA	TCTCTTAGAA	CCACAACAGT	CACAGGAAAG	ACAATCCATG	AATGGTGCTG
CA . . . . G . . C	. . T . A	. A . . C . . T . C	. T . . . . A	CTC . . AACG .	. . . . T . .
AA . . . . C . . .	. AT . G	. A	GT . . . . G . . .	TTG . . A . . C .	. . . . T . .
. A . . . . C . .	. . T . G . . G .	. . . . C . . T . C	AT . T . . . . . A	CT . G . . ACGC	. . AT . GT . TG
TAGATCTTGC	ACGTTACCCC	CCCTACGTTT	CAAAGGAGAA	GACGGGTGCT	GGTACGGCAT
. C	. . AC . . . . A .	. A . . . A . A . A	. . G . . . T . . G	. . T . . A	. . . . G . .
CC . C . . G . .	. . AC . T . . T .	. . . . G . . A . A	. . TG	. . . . . C	. . . . T . . . . .
CC . C . . C . . .	. . A . G . . T .	. . T . . A . G . .	. TTG	. . T	. . . . T . . G . .
GGAAATCAG 3400					
. . . . 3420					
. . . . 3421					
. . . G . . T . . 3420					

* Superscript numbers indicate the nucleotide base map positions using published sequences for the dengue viruses.[19-21] Bold sequences correspond to primer binding sites. Underlined sequences correspond to serotype-specific probe binding sites. Homologies with the sequence of dengue-1 virus are dotted. DEN-1 = dengue-1; DEN-2 = dengue-2; DEN-3 = dengue-3; DEN-4 = dengue-4

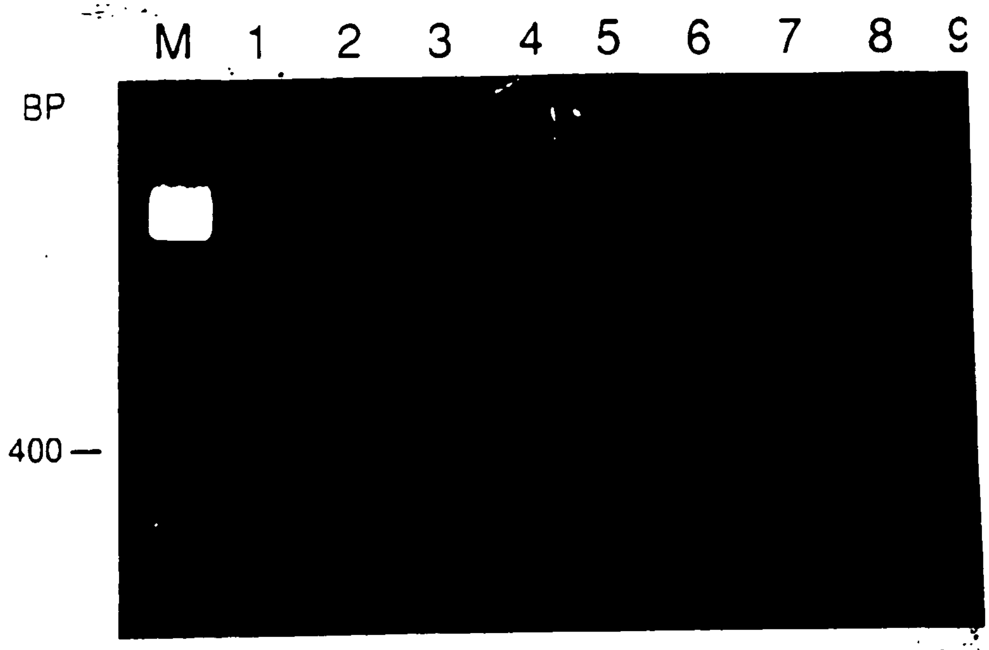

FIGURE 1. Analysis of polymerase chain reaction amplification products separated by electrophoresis on 1.2% agarose gel for one hr at 100 V and stained with ethidium bromide (0.05 μg/ml). Lane M, 123-base pa standard; lane 1, dengue-1; lane 2, dengue-2; lane 3, dengue-3; lane 4, dengue-4; lane 5, Japanese encephaliti lane 6, yellow fever; lane 7, Kunjin; lane 8, St. Louis encephalitis; and lane 9, uninfected cell culture supernatan

mM.[38] Digoxigenin-labeled probes were diluted to 0.05 μg/ml in hybridization buffer before use.

Nucleic acid hybridization

Forty microliter aliquots of each amplified DNA sample were treated with 4 μl of 3 M sodium hydroxide and incubated for 30 min at 70°C. Samples were neutralized with two volumes of 2 M ammonium acetate, vortexed, and cooled on ice. A one-fourth volume aliquot of each sample was slotted onto four separate Hybond-N nylon membranes using a Schleicher and Schuell (Keene, NH) slot-blotting apparatus. The DNA was crosslinked using a Stratalinker uv-box (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Membranes were treated with an excess volume of prehybridization buffer (6× SSC [1× SSC = 0.15 M sodium chloride, 0.015 M sodium citrate], 5× Denhardt's solution, [1× Denhardt's solution = 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin], 0.5% sodium dodecyl [illegible] 15% sodium pyrophosphate and 0.1 mg/ml sheared salmon sperm DNA) for two hr at 37°C in a heat-sealed bag. Membranes were hybridized with labeled oligomeric probes overnight at 37°C in hybridization buffer (6× SSC, 1× Denhardt's solution, 0.5% sheared salmon sperm DNA, 0.15% sodium pyrophosphate). Membranes were washed twice with an excess of 2× SSC, 1% sodium dodecyl sulfate for 15 min at ambient temperatures, twice with 2× SSC, 0.1% sodium dodecyl sulfate for 15 min at 37°C, and twice with 2× SSC, 0.1% sodium dodecyl sulfate for 15 min at 42°C.

Washed membranes hybridized with ^{32}P-labeled probe were air-dried and exposed to Kodak X-OMAT XAR-5 film (Eastman Kodak, Rochester, NY) and a Cronex lightning plus intensifying screen (Dupont). Reactions on washed membranes hybridized with non-isotopically labeled probes were detected using the chemiluminescent substrate, Lumiphos 530 (Boehringer Mannheim), according to the manufacturer's directions. Chemiluminescent reactions were recorded by exposing x-ray films to treated membranes for 3–15 min

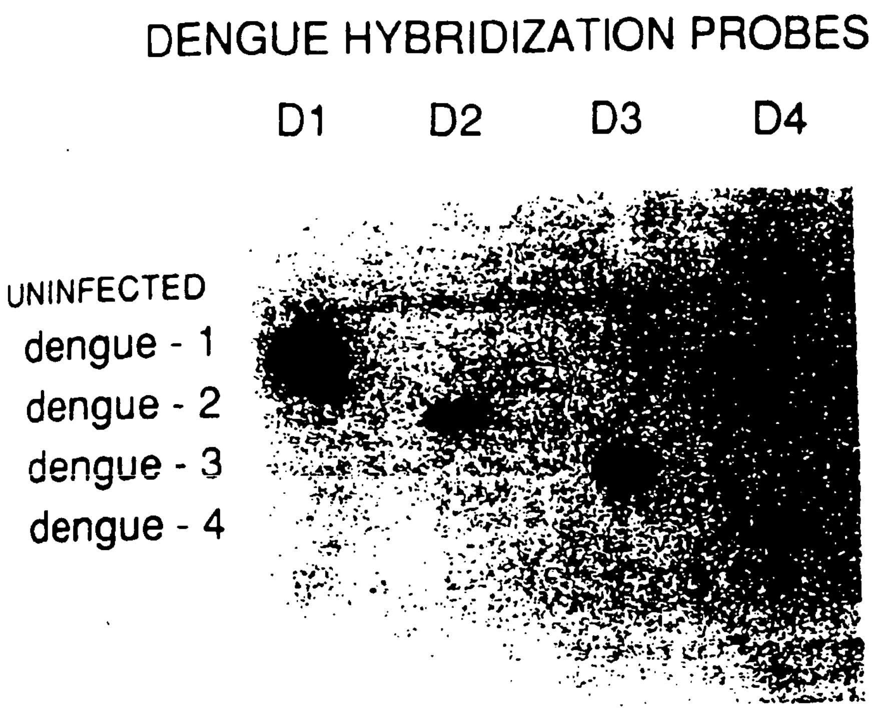

FIGURE 2. Autoradiograph of slot-blot hybridization of ^{32}P-radiolabeled, dengue-serotype specific oligomeric ...bes to amplified DNA using RNA extracted from uninfected and dengue-1, -2, -3, and -4 infected cell cultures. ...ne D1, dengue-1 specific; lane D2, dengue-2 specific; lane D3, dengue-3 specific; and lane D4, dengue-4 ...ific probes.

...ata analysis

The sensitivity and specificity of the assays were calculated by standard methods. Ninety-...e percent confidence intervals (95% CI) were ...culated by binomial approximation.[19]

RESULTS

...ificity and sensitivity using control virus sus-...nsions

In order to test the specificity of the universal dengue primer system, infected and uninfected (negative control) cell culture supernatants or suckling mouse brain suspensions were assayed ... RT PCR. The following viruses were tested: dengue-1, dengue-2, dengue-3, dengue-4, yellow fever, Kunjin, and St. Louis encephalitis virus. The PCR-amplified products are compared on an ethidium bromide-stained 1.2% agarose gel ... size of the amplified DNA was consistent with that predicted for the target region in the dengue viral genomes (approximately 400 base pairs). Ethidium bromide-reactive, non-specific material smaller than amplified DNA was occasionally obtained with suckling mouse brain preparations.

Dengue virus serotypes were identified by nucleic acid hybridization of serotype-specific oligomeric DNA probes with amplified cDNA. Slot-blot hybridization was chosen as a method of evaluation because it allowed for simultaneous serotype identification using four different synthetic probes. Amplified cDNA from uninfected or dengue-1, dengue-2, dengue-3, or dengue-4–infected cells were each hybridized against serotype-specific, oligomeric DNA probes (D1, D2, D3, and D4) (Table 1). Each oligomeric probe reacted only with the homologous virus serotype (Figure 2).

... neutralizing human

RT/PCR detection of dengue virions, a reference stock of dengue-2 (4.7 × 10^6 pfu/ml) was diluted 1:100 and then two-fold in phosphate-buffered saline or pooled human serum containing high-titered, anti-dengue antibodies. One-hundred microliter aliquots of each dilution were then assayed by RT/PCR. Amplified DNA was Southern blotted and hybridized using radiolabeled D2 probe (Figure 3). The last electrophoretic bands visible in the absence (top panel, lane 5) or presence (bottom panel, lane 6) of pooled human anti-flavivirus antiserum are equivalent to 4.4 and 9 pfu, respectively. The presence of anti-viral antibodies did not lower the sensitivity of the method. The two-fold increase in sensitivity shown using human serum as a diluent was not reproducible.

Patient specimens

Sera from 74 patients with dengue and non-dengue or virus-negative control sera assayed using RT/PCR. Amplified DNA wa characterized by agarose gel electrophoresi staining with ethidium bromide, followed by blot nucleic acid hybridization. Forty-two imens were tested using both radioisotopic chemiluminescent detection methods. Th sults in Table 3 show the sensitivity and s ficity of the three assays for identifying de virus patients using admission sera, wheth not a virus isolate had been obtained. Slot- nucleic acid hybridization using radiolab probes appeared to be the most sensitive a (68%, 50/74; 95% CI = 57–78%). Virus isol

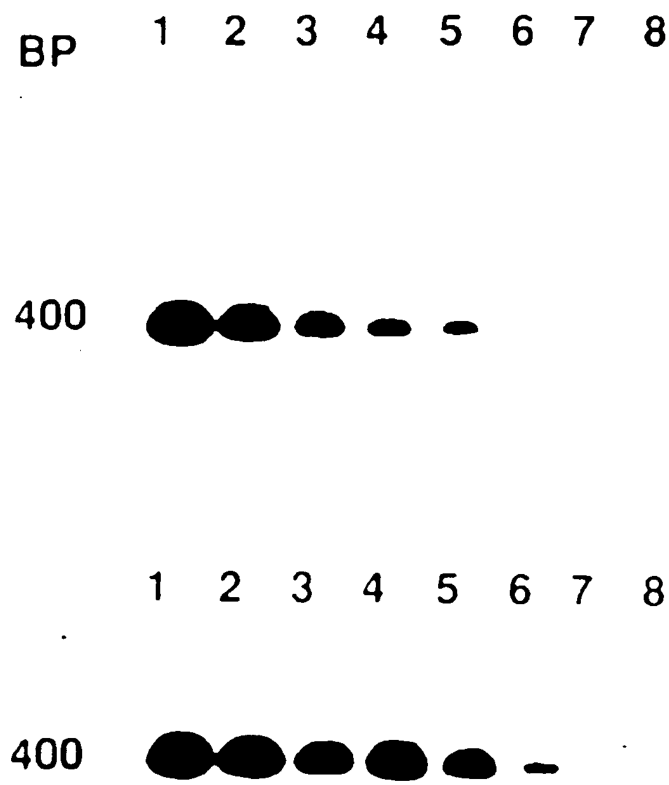

FIGURE 3. Sensitivity of the reverse transcriptase-polymerase chain reaction assay to detect dengue-2 virus in the presence or absence of human sera containing anti-flavivirus antibodies. (Top panel), virus culture supernatant only. (bottom panel),, virus culture supernatant diluted with pooled human anti-flavivirus serum. Lane 1, 1:20 dilution; lane 2, 1:200; lane 3, 1:400; lane 4, 1:800; lane 5, 1:1, 600; lane 6, 1:3, 200; lane 7, uninfected cell culture material; and lane 8, diluent only.

TABLE 3

...ity and specificity of the polymerase chain reaction assay for detecting dengue viruses in acute human sera

	Ethidium bromide		Slot-blot nucleic acid hybridization*			
			Radiolabeled probes		Non-isotopically labeled probes	
Result	Dengue	Non-dengue	Dengue	Non-dengue	Dengue	Non-dengue
Positive	40	1†	50	0	26	0
Negative	34	8	24	9	17	9
Total	74	9	74	9	42	9
Sensitivity	0.54		0.68		0.62	
Specificity	0.89		1.00		1.00	

* ...otted amplified DNA was hybridized with radiolabeled or non-isotopically labeled (using a chemiluminescent substrate) dengue-specific ... Hybridization reactions were recorded on x-ray film.
† Negative upon subsequent retesting.

...were not obtained from 10 of 50 RT/PCR-positive sera from serologically confirmed dengue patients. In comparison, detection of amplified DNA by ethidium bromide staining of agarose gels had a sensitivity of 54% (40/74; 95% CI = 43–65%). Chemiluminescent detection using a smaller number of specimens had a sensitivity of 62% (26/43; 95% CI = 46–75%). Exposure of x-ray films to chemiluminescent reactions longer than 15 min resulted in high background levels with decreasing sensitivity (unpublished data). The false-positive reaction detected using ethidium bromide staining was negative upon retesting and by other forms of the assay.

Using specimens from which a dengue virus had been isolated, the performance of the assays with RNA from each of the four dengue virus serotypes was examined. These results are summarized in Table 4. To calculate sensitivity, a true positive was defined as a culture-positive specimen; to calculate specificity, a true negative was defined as a culture-negative specimen. Slot-blot hybridization using radiolabeled probes appeared to be slightly more sensitive than alternative forms of the assay. Detection of amplified products by nucleic acid hybridization with radiolabeled probes resulted in sensitivity values ranging from 60% to 100%, while other assays were sensitive in the range of 14–100%. For all dengue viruses, RT/PCR followed by nucleic acid hybridization with radiolabeled probes was 80% sensitive (40/50; 95% CI = 69-91%) and 100% specific. Specimens that were RT/PCR-positive and false-negative specimens generally contained similar amounts of virus by mosquito inoculation (10^3–10^4 MID_{50}). False-positive results were obtained using ethidium bromide staining (one non-dengue specimen was identified as dengue-2) and chemiluminescent detection of hybridization products (one specimen containing dengue-4 virus was falsely identified as dengue-2).

DISCUSSION

Previous investigators have described an eight primer mixture for serotype-specific PCR am-

TABLE 4

...ity and specificity of the polymerase chain reaction assay for dengue viruses of known serotype in human ...

	Ethidium bromide		Slot-blot nucleic acid hybridization*			
			Radiolabeled probes		Non-isotopically labeled probes	
...type†	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Dengue-1 (8)	0.50 (4)‡	1.00	0.75 (6)	1.00	0.14 (1)	1.00
Dengue-2 (31)	0.71 (22)	0.96	0.87 (27)	1.00	0.75 (18)	0.96
Dengue-3 (1)	1.00 (1)	1.00	1.00 (1)	1.00	0.00 (0)	1.00
Dengue-4 (10)	0.60 (6)	1.00	0.60 (6)	1.00	0.60 (6)	1.00
All (50)	0.66 (33)	0.89	0.80 (40)	1.00	0.60 (25)	1.00

* ...otted amplified DNA was hybridized with radiolabeled or non-isotopically labeled dengue-specific probes. Hybridization reactions were

plification of dengue virus RNA, and serotype identification using cloned, unlabeled cDNA probes in nucleic acid hybridization assays.[21] In this study, we evaluated PCR amplification of dengue virus RNA using a universal primer set, followed by nucleic acid hybridization with radiolabeled or non-isotopically labeled synthetic oligomeric probes. Primer sequences were based upon degeneracy in codon "wobble" bases. This approach has been previously suggested for amplification of coding regions with small patches of sequence similarity for uncharacterized retroviruses.[40–42] By constructing degenerate primers, we were able to use a limited amount of sequence information to construct dengue-specific primers for the RT/PCR assay. Since degeneracy can be built into oligomeric sequences at the time of synthesis, only a single set of primers was needed to amplify dengue virus cDNA, regardless of the serotype present.

Since the universal primer set was designed to amplify all dengue viruses, a slot-blot nucleic acid hybridization assay using serotype-specific oligomeric DNA probes was necessary for serotype identification. This is the first time that a system of serotype-specific, oligomeric DNA probes for use in nucleic acid hybridization assays has been described for all four dengue virus serotypes. Other investigators have used cloned gene fragments as much as 10-fold larger than our proposed probes.[22] Synthetic oligomeric probes may have wider applicability for the field laboratory.

The presence of high-titered anti-dengue antibodies in serum has an adverse effect on virus isolation or detection using solid-phase capture assays.[6,43] Innis and others showed that dengue virus isolation was more common in IgM-negative than in IgM-positive acute sera.[6] Our studies and those of others[22] confirm that detection of dengue viruses can be accomplished, even in the presence of high-titered neutralizing antibodies. These results suggest that RT/PCR will be an important adjunct to the laboratory confirmation of dengue virus infection.

Coupled with nucleic acid hybridization of amplified products with non-isotopically labeled probes or radiolabeled probes, RT/PCR was 62% and 68% sensitive for all dengue patients, respectively. These results are consistent with other diagnostic assays using admission sera: HI (53%), dengue IgM (10–78%), and virus isolation (20–80%).[2,6] The method we evaluated performed similarly to the assay described by bel and others, which had an estimated sen ity of 75%.[22] Large comparative trials wi required to identify the best RT/PCR assay tem. Among those specimens for which a isolate had been made, this method had an c all sensitivity of 80%. Dengue-4 viruses ha lowest degree of sensitivity to detection (6 and this may be due to unrecognized sequ variations in the amplified regions.[2,37]

The occurrence of false-negative results not correlate with specimens with lower v titer. Some false-negative results may have curred because of repeated freeze/thaw cy prior to RT/PCR testing. Other causes of fa negative results remain to be discovered. Th results suggest that virus amplification in mosquito remains a superior, yet time-consu ing, diagnostic method.

In these experiments, digoxigenin-labe probes were less sensitive than radioactively beled probes. In the case of dengue-1 virus unlabeled probes reacted with only one of ei amplified cDNA specimens (14%), while det tion by ethidium bromide staining or radioact probes were 50% sensitive (four of eight) a 75% (six of eight), respectively. Since only sm numbers of virus isolates were available for so serotypes, additional studies with larger numb of specimens and broader panels of isolates required.

The RT/PCR was 89–100% specific, regar less of whether a virus isolate was obtained. Sin other diagnostic methods can not make an e ologic diagnosis without a virus isolate, the results are remarkable. The use of radiolabele serotype-specific probes allowed for an additio al level of specificity (96–100% specific) u available in assays using RT/PCR alone (8 100%). Some cross-reactions obtained with no isotopically labeled, synthetic probes may be t result of the addition of extraneous nucleotic bases during labeling or non-specific enzymat reactions. Further development may be require to improve non-isotopic labeling methods.

Our successful application of non-isotop methods to the detection of the dengue viruse paves the way for adaptation of the method i routine diagnostic laboratories. We envision th ultimate processing of a clinical specimen to in clude amplification of genomic sequences wit carefully chosen primer sequences, as well as th detection of dengue virus-specific IgM. The RT

...R-positive sera can be later cultured if recov... of virus is desirable for research or reference purposes.

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Authors' addresses: Erik A. Henchal, Military Disease Hazards Research Program, SGRD-PLA, US Army Medical and Development Command, Frederick, MD ...-5012. Stephanie L. Polo and Charles H. Hoke, Department of Virus Diseases, Walter Reed Army Institute of Research, Washington, DC 20307-5100. ...ance Vorndam, Centers for Disease Control, San Juan, PR 00936. Chandhana Yaemsiri and Bruce L. Innis, Department of Virology, Armed Forces Research Institute of the Medical Sciences, APO San Francisco, ... 96346-5000.

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Identification of dengue sequences by genomic amplification: rapid diagnosis of dengue virus serotypes in peripheral blood

Vincent Deubel[1], Manola Laille[2], Jean-Philippe Hugnot[1], Eliane Chungue[3], Jean-Luc Guesdon[4], Marie Thérèse Drouet[1], Sylviane Bassot[1] and Danièle Chevrier[4]

[1]*Institut Pasteur, Laboratoire des Arbovirus, Paris, France,* [2]*Institut Pasteur de Nouvelle Calédonie, Laboratoire des Arbovirus, New Caledonia,* [3]*Institut Territorial de Recherches Médicales Louis Malardé, Laboratoire de Virologie, Papeete, Tahiti, French Polynesia and* [4]*Institut Pasteur, Laboratoire des Sondes Froides, Paris, France*

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Summary

Polymerase chain reaction (PCR) was developed for the in vitro amplification of dengue virus RNA via cDNA. A fraction of the N-terminus gene of the envelope protein in the four dengue serotypes was amplified using synthetic oligonucleotide primer pairs. Amplified products were cloned and used as dengue type-specific probes in gel electrophoresis and dot-blot hybridization. We detected and characterized dengue virus serotypes in blood samples by the three-step procedure DNA-PAH consisting in cDNA priming (P), DNA amplification (A) and hybridization (H) using specific non-radiolabelled probes. Our findings showed that DNA-PAH was more rapid and sensitive in the identification of the infecting serotype than the mosquito cell cultures. Moreover, the failure of cultures to detect virus particles in sera containing few copies of viral genome or anti-dengue antibodies justified the approach of DNA-PAH to the dengue identification in clinical specimens.

Dengue virus; Polynucleotide chain reaction; Virus diagnosis

Correspondence to: Vincent Deubel, Laboratoire des Arbovirus, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France.

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Introduction

The flavivirus genus contains approximately 70 related members classified in subgroups including dengue, yellow fever, Japanese encephalitis and tick-borne encephalitis. Their genome is a single-strand positive sense RNA of about 11 000 nucleotides which lacks a poly(A) tract at its 3′-extremity. In terms of morbidity, dengue fever is the most important mosquito-borne viral disease of man, causing in tropical latitudes millions of cases annually (Halstead, 1988). Each of the four dengue serotypes can be genetically (Blok et al., 1989; Chu et al., 1989; Trent et al., 1989) and antigenically (Monath et al., 1986) subdivided into subtypes that may vary in virulence (Rosen, 1987).

The extent of dengue virus circulation and the increasing rate of more severe symptoms in children known as dengue haemorrhagic fever/dengue shock syndrome cause a major problem of public health (Schlesinger, 1977). The rise of epidemics outside the Southeast Asian endemic part of the world results from the introduction of new variants and serotypes, mainly by means of air travel. Therefore, rapid characterization of the causal agent would help institute measures to control outbreaks. Moreover, the identification of the variations and evolution of the viral genome would contribute to our understanding of the epidemiology and the molecular aspects of the disease.

The clinical and epidemiological studies on dengue are based upon serological diagnoses that are time-consuming or non specific (Burke et al., 1988) and virus isolation (Kuno et al., 1985). Recovering virus from viremic sera might fail if the specimens contained anti-dengue antibodies or were improperly preserved. The virus serotype can usually be determined by using type-specific monoclonal antibodies to detect the virus envelope protein in infected cultured cells (Henchal et al., 1982). Dengue 2 virus has been identified in specimens by specific hybridization using cDNA probes (Henchal et al., 1987; Khan and Wright, 1987). However, direct detection with nucleic acid probes from blood samples is limited by the low viremia in some samples and the need for specific probes corresponding to each serotype. The recent publication of the sequence of the structural genes (Deubel et al., 1986; Mason et al., 1987; Osatomi et al., 1988; Zhao et al., 1986) allows the preparation of such probes. The development of the polymerase chain reaction (PCR) allowed a selective and rapid amplification of short segments of genome (Saiki et al., 1988). It has proved its utility in the detection of viral pathogens (Persing and Landry, 1989) and its power to acquire direct sequences from the amplified gene molecules (Engelke et al., 1988). In this study, we have developed a rapid and sensitive diagnostic tool for identification of dengue viruses in serum specimens by cDNA amplification coupled to hybridization with type-specific cDNA probes (DNA-priming-amplification-hybridization or DNA-PAH).

Materials and Methods

Cells and viruses

Dengue virus strains used in this study (dengue 1: 331-98, Tahiti 1988; dengue 2: 1409, Jamaica 1983; dengue 3: PaH 881, Thailand 1988; dengue 4: PaH 813, Martinique 1981) were isolated from human sera and propagated on C6/36 (*Aedes albopictus*) cell monolayers. The serotype was confirmed by indirect fluorescent antibody tests with type-specific monoclonal antibodies (Henchal et al., 1982).

Human sera

Sera used in the comparative diagnosis were collected from patients who had a dengue illness confirmed by virus detection or serological investigation. Serological confirmation of dengue was achieved by demonstration of IgM (Chungue et al., 1989) in an early sample or, if negative, in a convalescent sample collected 2 weeks later. The acute sera were collected within 5 days after the onset of the fever. Except for one case of dengue 2, samples were from humans infected with dengue 1, 3 or 4 during dengue epidemics in 1988–1989 in the South Pacific. Information to determine whether the patients sustained primary dengue or dengue superinfection was not available.

Virus growth, RNA extraction and cDNA synthesis

Dengue viruses were grown in C6/36 cells (m.o.i.=1-5 PFU/cell) for 3 days in Eagle's minimum essential medium containing 2% fetal calf serum. Cells were washed in 1 × TNE (Tris/NaCl/EDTA) buffer and lysed in 0.1 × TNE containing 0.5% NP40. Cell nuclei were pelleted by low speed centrifugation and the cytoplasmic extract was deproteinized with 3 treatments of phenol in presence of 1% SDS. To recover RNA from serum, 200 μl of sample were incubated for 1 h at 56°C in presence of 0.5% SDS, 10 μg proteinase K (Boehringer) and 80 units RNAse inhibitor (Promega). Sample was diluted with 300 μl TNE buffer and subjected to 2 phenol treatments. RNA was precipitated with 2.5 volumes of pure ethanol/0.3 M ammonium acetate.

Five μg of intracellular RNA or RNA corresponding to 50 μl of serum sample were primed with oligonucleotide primers for first strand cDNA synthesis using reverse transcriptase (Deubel et al., 1986). Four oligonucleotide primers were synthesized complementary to E gene sequences of dengue 1 (strain CV1636/77), dengue 2 (strain 1409), dengue 3 (strain H87) and dengue 4 (strain 814669) viruses (Chu et al., 1989; Deubel et al., 1986; Osatomi et al., 1988; Zhao et al., 1986), respectively (Table 1). Dried RNA was dissolved in 10 μl of water with 100 ng of oligonucleotide. The mixture was heated at 90°C for 2 min, then cooled on ice. cDNA synthesis was performed in 20 μl of Tris.HCl 50 mM, pH 8.3, KCl 50 mM, $MgCl_2$ 8 mM, dithiothreitol 10 mM, 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.5 mM dTTP, 40 units RNAse inhibitor and 2 units

TABLE 1
Sequences of synthetic oligonucleotide primers

Primer[a]	Number[b]	Sequence[c]	Length of the PCR product (bp)
D1(+)	52	GCAACGTGGGTTGACGTGGTATTGG	237
D1(−)	288	AAACGTTCGTCTACACACAAAGTTCG	
D2(+)	39	GGGGTTTCAGGAGGAAGCTGGGTTGAC	266
D2(−)	305	CCCCATCCTCTGTCTACCATG	
D3(+)	47	CGGGAGCTACGTGGGTTGACGTGG	257
D3(−)	303	CCAGCCTCTGTCTACGTATGTATGC	
D4(+)	40	GGAGTCTCAGGTGGAGCATGGGTCGAC	267
D4(−)	306	GCCCCACCCTCTGTCTACCACATC	

[a]D1,2,3,4: primer of dengue serotype number 1,2,3,4 of genomic (+) or anti-genomic (−) sense.
[b]Number indicates the map site at which the 5'-end of the oligonucleotide hybridizes on the strain gene E.
[c]Mismatch in the nucleotide sequence for site-directed mutagenesis is underlined.
bp: base pairs.

reverse transcriptase (Boehringer). The reaction was incubated for 1 h at 42°C and stopped in 0.2 M EDTA. DNA was phenol extracted and ethanol precipitated.

cDNA amplification

For standard PCR (Saiki et al., 1988), one fifth of the cDNA product was added to a final volume of 50 μl containing 50 mM KCl, 10 mM Tris.HCl pH 8.4, 2 mM $MgCl_2$, 10 μg gelatin, 250 μM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP), and 0.3 μg of each oligonucleotide primer. The sequences of the primers corresponding to the viral genomic sense were chosen at about 250 nucleotides upstream from those of the primers used for cDNA synthesis (Table 1). First, a denaturation of the RNA-cDNA hybrids was done at 95°C for 10 min followed by primer annealing for 2 min at 55°C. 2.5 units of *Thermus aquaticus* (*Taq*) polymerase (Perkin-Elmer Cetus) were added and the reaction was maintained at 72°C for 90 s. Amplification was achieved by 36 cycles with the following step cycle: denaturation at 94°C for 15 s, annealing at 55°C for 1 min, and extension at 72°C for 45 s. After the last cycle, samples were maintained at 72°C for 12 min. Five μl of each reaction mixture were electrophoresed through 1.5% agarose gel in TBE (Tris/Borate/EDTA) stained with ethidium bromide for DNA visualization.

Cloning of dengue cDNA

One half of the amplified DNA mixture was incubated with restriction enzymes *Hinc*II and *Acc*I (Table 1), then loaded on a 1% low melting point agarose gel

(Gibco Bethesda Research Laboratory) in TAE buffer (Tris/acetate/EDTA). The DNA was excised from the gel, phenol extracted, and ethanol precipitated in 2.5 volumes of pure ethanol/0.3 M NaCl. The DNA was cloned into the *Eco*RV- and *Hinc*II-cut, calf intestine phosphatase-dephosphorylated plasmid bluescript pKS(-) (Alting-Mees and Short, 1989; Maniatis et al., 1982). Dengue double-stranded recombinant DNA was sequenced using the M13 universal primer and T7 polymerase (Sequenase, US Biochemicals).

Southern and dot-blot hybridization

DNAs from agarose gel were transferred to cellulose nitrate membrane by the Southern method (Maniatis et al., 1982). For dot hybridization, samples of DNA corresponding to one tenth of the PCR product were denatured in 50 mM Tris.HCl, pH 7.4, 0.2 N NaOH, 6 × SSC for 10 min at 80°C. After neutralization with 0.2 M Tris.HCl, pH 7.4, the samples were spotted onto nitrocellulose filters by using a manifold (Minifold I, Schleicher & Schuell). The membranes were heated for 2 h at 80°C and prehybridized for 30 min at 42°C in a buffer containing 4 × SSC, 5% powdered milk, 50% formamide, 0.1% SDS, then hybridized overnight at 37°C in the same solution containing the labelled dengue-specific probe. The recombinant plasmids containing each a fraction of E gene corresponding to a dengue serotype were labelled with [^{32}P]dCTP (Amersham) using the nick-translation procedure (Boehringer) and were used as radioactive probes for hybridization tests. Membranes were washed (Maniatis et al., 1982), dried and exposed at −70°C to X-ray film (Hyperfilm-MP, Amersham) using an intensifier screen. Nonradioactive probes were prepared by labelling with a N-acetoxy-N-2-acetylaminofluorene (AAAF) and used for DNA-DNA hybrid detection with anti-AAF monoclonal antibody as previously described (Chevrier et al., 1989). Briefly, the filter was hybridized in the same condition as above with 200 ng of heat-denatured AAF-labeled DNA probe per ml. After washing, the filter was incubated for 1 h with anti-AAF monoclonal antibody, washed and incubated for 1 h with alkaline phosphatase-labelled sheep anti-mouse IgG antibody. The enzyme on positive hybridization was revealed for 30 min with a mixture of 15 ml of 0.33 mg/ml of Nitro Blue Tetrazolium and 15 ml of 0.16 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris.HCl, pH 9.5, 100 mM NaCl, 50 ml $MgCl_2$.

Results

Selection, amplification and evaluation of the dengue E gene fragments for specific hybridization

The secondary structure of the envelope protein E of the flaviviruses is highly conserved and contains 3 major antigenic domains, A, B and C (Mandl et al., 1989). Domain A located at the N-terminus of the protein E is stabilized by 3

Fig. 1. Amplification products. RNA from cells infected with dengue 1 (1), dengue 3 (3) or dengue 4 (4) virus was amplified by PCR using specific primer sets (Table 1). Plasmid p30-VD2 (Deubel et al., 1986) was digested with *Acc*I and *Sca*I (MW) or with *Hin*cII and *Acc*I (2). Shown is the ethidium bromide-stained 1.5% agarose gel. Arrowhead indicates the amplified DNA fragment.

disulfide bridges and the majority of its antigenicity is destroyed by detergent and low pH (Guirakhoo et al., 1989). In this domain, a hydrophilic peptide ranging from amino acid 22 to 97 contains a linear epitope (Deubel, unpublished results), one neutralizing epitope (Mandl et al., 1989; Lobigs et al., 1987) and a glycosylation site. The corresponding nucleotide sequence shows great changes between variants belonging to the same serotype (Blok et al., 1989; Chu et al., 1989). Moreover, this gene fragment is flanked by sequences relatively conserved between geographical variants that have been used as primer for gene amplification. Two restriction sites, *Hin*cII and *Acc*I, natural or introduced by PCR (Table 1), were used to clone the amplified fragments (Fig. 1). Clearcut PCR products of about 250 base pairs (bp) were obtained with each set of primers used for dengue 1, 3 and 4 gene amplification, respectively, and were compared to the product of *Hin*cII-*Acc*I-digested plasmid p30-VD2 (Deubel et al., 1986) containing the structural genes for dengue 2 virus (Fig. 1).

The identity of the product was confirmed by cloning and sequencing the amplified DNA trimmed at its extremities with *Hin*cII and *Acc*I and corresponding to 227 bp (213 bp for dengue 1) (Fig. 2). The four dengue sequences showed 32 to 39% of nucleotide divergence. The *Hin*cII-*Acc*I cloned nucleotide fragments in the gene E of dengue viruses were used for hybridization tests.

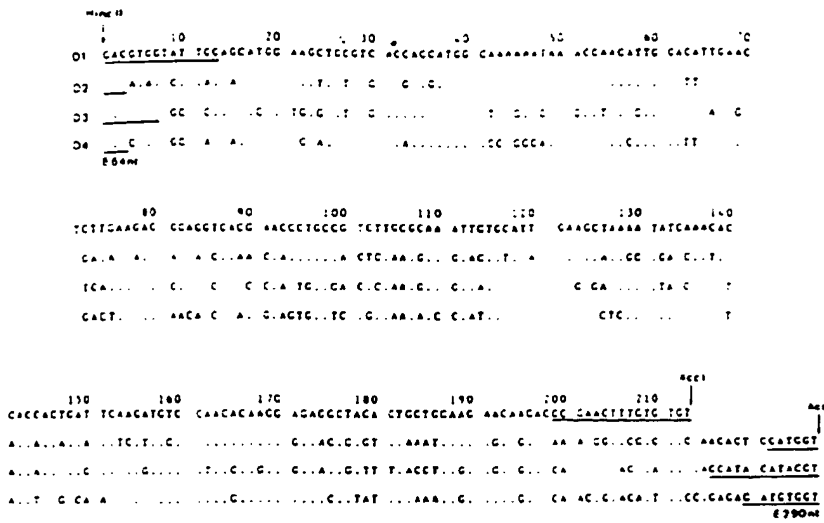

Fig. 2. Hybridization probe sequences. cDNA fragments of the envelope protein gene for the four dengue serotypes (indicated as 1, 2, 3 and 4) were cloned in pKS bluescript plasmid and were sequenced (see Materials and Methods). *HincII* and *AccI* cloning sites are indicated. Homologies in the nucleotide sequences with the sequence of dengue 1 virus are dotted. Overlapping sequences with the primers are underlined. Localization of the fragments in the dengue envelope gene are indicated by first and last nucleotides (nt).

Evaluation of the specificity of the DNA-PAH for dengue serotype determination

A mixture of the 4 dengue-specified anti-genomic oligonucleotides (Table 1) was used to prime RNA extracted from cells infected by each of the dengue serotypes, respectively. The cDNAs synthetised were then amplified by PCR using a mixture of four pairs of dengue-specific primers (Table 1). Fig. 3A shows the amplified DNAs in the ethidium bromide-stained gel. Each lane corresponded to the product of one dengue serotype which had infected the cells. In lane C, product from mock-infected cells was used as control. Several bands of different intensity are seen in each lane. Nevertheless, a band of high intensity for the 3 first serotypes and of lower intensity for the dengue 4 and the control is obvious at the expected size of about 250 bp. The specificity of the DNA bands was confirmed by Southern blot hybridization with (α-^{32}P)dCTP nick-translated probes. The probes corresponded to double-stranded recombinant plasmids containing the dengue inserts shown in Fig. 2. Fig. 3B shows that the PCR product of RNA from each dengue serotype is specifically recognized by the corresponding probe. Moreover, bands of a size larger than expected are also recognized. The dengue 1 probe shows a slight cross-reactivity with the dengue 3 cDNA when the membrane is washed at 42°C. On the other hand, no bands from mock-infected cells were recognized by any of the probes.

To circumvent the time-consuming and laborious technique of Southern blot and the use of radiolabeled probes, we have dotted on nitrocellulose the same

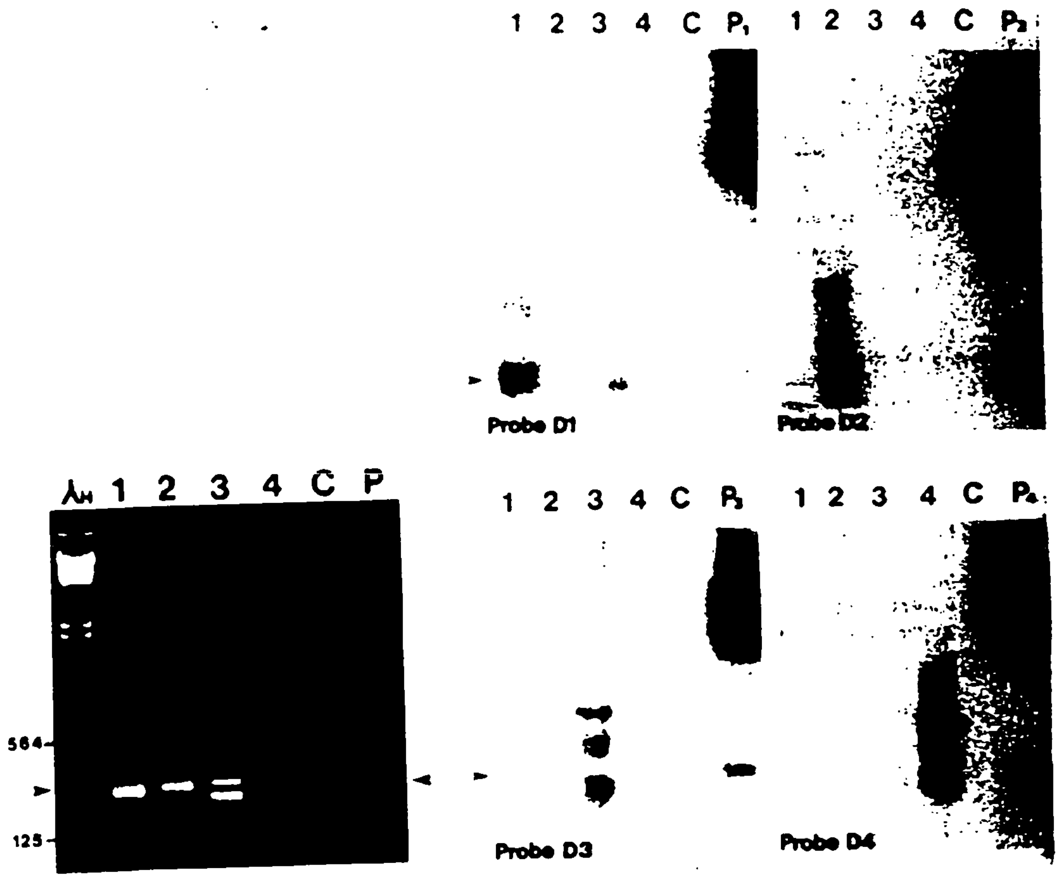

Fig. 3. Identity of products amplified from RNA of the 4 dengue serotypes. A mixture of dengue primers was used for PCR. Left: ethidium bromide-stained 1.5% agarose gel. Right: autoradiograph of Southern transfer from gel, hybridized to the dengue (D)-specified probes. Washing procedures were performed at 42°C. Amplification products of RNA isolated from cells infected with dengue 1 (1), dengue 2 (2), dengue 3 (3), dengue 4 (4), or from mock-infected cells (C). P1,2,3,4 correspond to the dengue-specified plasmids used as probe: 100 ng were digested with *Hinc*II and *Acc*I for molecular size determination (indicated by arrows) and hybridization control. (λH): *Hind*III-cut lambda DNA.

products of DNA amplification as those shown in Fig. 3. The dengue serotype was determined by using the same four probes labeled with AAF (see Materials and Methods). The DNA-DNA hybridization data shown in Fig. 4 confirm the specificity of the dengue probes for the amplified DNA.

Evaluation of the sensitivity of the PCR

We have tested the sensitivity of the PCR for the detection of dengue virus genome by mixing serial dilutions of viral particles titrated on *Toxorhynchites*

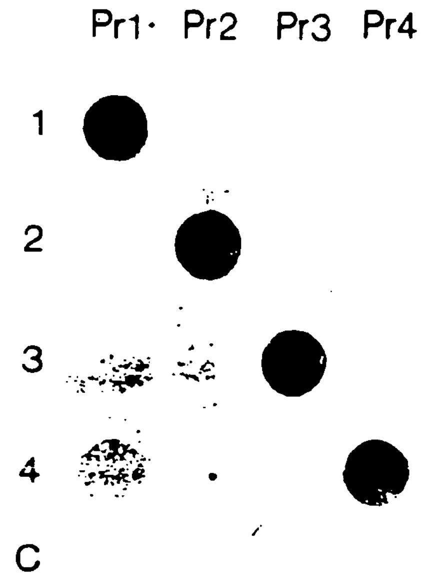

Fig. 4. Specificity of spot hybridization assays. Rows 1,2,3,4 contained DNA amplified from each dengue serotype (see legend figure 3). AAF-labelled probes specific to each dengue serotype are indicated Pr1, Pr2, Pr3 and Pr4, respectively. (C): RNA amplified from mock-infected cells. Membrane washing was carried out at 65°C.

mosquitoes (Rosen and Gubler, 1974), with normal serum. These mixtures served either for mosquito cell culture inoculation or for RNA extraction and PCR procedure. Amplification products were shown when at least 1 viral particle was present in the serum sample (Table 2). As DNA-DNA hybridization was performed on the equivalent of 1 μl of serum we can expect that it detects at least a viremia of 10^3 infecting particles per ml. Dengue viruses from clinical samples are most commonly characterized after culture on mosquito cells (Kuno et al., 1985). In order to compare PCR with cell culture, 24-well plates of C6/36 cells were inoculated with 50 μl of serum samples containing increasing titers of dengue 2 viral particles (Table 3). The results show that the limit of virus detection in culture corresponds to a viremia about 2×10^4 mosquito-infecting particles per ml and that it requires 6 days (Tables 2 and 3).

Detection of dengue virus RNA in peripheral blood of infected patients

We have applied the DNA-PHA to 32 specimens of blood from febrile patients who experienced a dengue illness. Anti-dengue IgM antibodies were detected in 8 of these samples (data not shown). The other sera seroconverted later on, as demonstrated by presence of IgM in a second serum sample (see Materials and

TABLE 2
Evaluation of different techniques for dengue 2 virus identification

	Volume of the sample (μl)	MIP[a]	Limit of detection (MIP/ml)	Days needed for detection
Intrathoracic inoculation in mosquito	2	1	500	10
PCR	1[b]	1	1,000	2
Cell culture	50[c]	1,000	20,000	6

[a]Number of mosquito-infecting-particle giving a positive signal in either test.
[b]cDNA of dengue 2 virus was amplified from serum sample using the set of dengue 2 primers (Table 1). Positive amplification was monitored by Southern blot hybridization using ^{32}P-labeled probe (see Materials and Methods).
[c]Data are derived from results shown in Table 3.

TABLE 3
Temporal study of dengue 2 virus replication in C6/36 cells

Virus titer[a]	Immunofluorescence score[b] (Days after infection)							
	3	4	5	6	7	8	10	11
10	–	–	–	–	–	–	–	–
10^2	–	(+)	(+)	–	(+)	+	–	(+)
10^3	–	(+)	(+)	+	+	+	+	+
10^4	–	(+)	+	+	+	+	+	+

[a]The titer corresponds to mosquito-infecting particles inoculated per well.
[b]Screening system: –, no infection; (+), less than 2% cells infected; +, many cells infected.

Methods). Dengue viruses were isolated in cell culture for 15 samples and the serotype was confirmed by immunofluorescence (Table 4). None of these sera contained anti-dengue IgM antibodies (data not shown). Dengue virus RNA was detected by DNA-PAH in serum from all the 15 patients in whom the virus had been isolated and in the serum of 9 patients found negative by culture. Three of these 9 sera carried anti-dengue IgM antibodies (data not shown). The serotype of the 24 dengue-positive specimens was determined by specific hybridization (Table 4) and was confirmed by using the corresponding type-specific pair of primers for a second DNA-PAH. In contrast, 8 specimens were negative for DNA-PAH in duplicated experiments. On the other hand, two samples collected from normal volunteers were chosen as negative internal controls. These sera were negative by DNA-PAH and cell culture.

Discussion

A rapid, sensitive and specific test for dengue virus RNA detection in infected specimens has been developed. Primer oligonucleotides corresponding to sequences of the four dengue serotypes have been selected for cDNA priming and amplification. We have also chosen sequences conserved in dengue topo-

infection with a second serotype. Disease known as dengue hemorrhagic fever accompanied by internal bleeding and shock (DHF/DSS) is frequently found in endemic Asian countries where case-fatality rates in children reach 2 to 10% (Halstead, 1988). These severe cases of dengue requiring hospital admission usually develop a few days after onset of fever, when the viremia is decreasing and low. The rapidity of the DNA-PAH test that could be performed in 2 days offers an advantage over standard tissue culture (Table 3) for virus detection. On the other hand, the higher sensitivity of DNA-PAH method compared to cell culture to detect dengue virus has been confirmed on viremic samples (Tables 3 and 4). First, we have analyzed samples in which the virus was titrated and we concluded that PCR was about 20 times more sensitive than cell culture (Tables 2–4). Second, we have been able to prime and to amplify DNA complementary to viral RNA in 9 samples found negative by culture (Table 4). In 8 sera, immune complexes may have inhibited the cell infectivity of the virus (data not shown). Other causes that could explain false-negative results in culture, are low titer of virus and preservation of the sample at inadequate temperature before the test. We found 24 dengue-positive sera by DNA-PAH among 32 patients in whom dengue virus infection was confirmed by a presence of anti-dengue IgM antibodies in the acute or convalescent serum samples. False negative results in PCR for 8 specimens obtained few days after the onset of fever are not yet understood. We were more successful in detecting dengue 1 genome in culture negative samples than dengue 3 and 4 (Table 4). Nevertheless, using our DNA-PAH test, we were able to amplify and to detect the genome fragment of several dengue genomic variants (Rico-Hesse, 1990), 4 of dengue 1, 7 of dengue 2, 3 of dengue 3 and 3 of dengue 4 (Deubel et al., manuscript in preparation). Therefore, more samples for virus isolation in epidemic areas have to be tested to appreciate the reliability of PCR over tissue culture.

Dengue virus replication in humans appears to be restricted to mononuclear cells (Halstead, 1988). Moreover, higher rates of virus isolation from peripheral blood leukocytes than from serum have been observed (Scott et al., 1980). It would be of interest to apply the PCR technique to these cells which might be increased in power for early diagnosis. A better understanding of the dengue pathogenesis could be provided by PCR while characterizing other putative target cells important for dengue infection.

PCR would improve the accuracy in viral sequence determination. Sequencing the gene directly amplified from clinical specimens would avoid variant strain selection or mutation event that may occur in tissue culture (Engelke et al., 1988). The PCR using *Taq* polymerase has an error rate of 0.8% mutation after 20 cycles (Keohavong and Thilly, 1989). This may not cause any problem for direct sequencing since misincorporation generally occurs uniformly along the amplified DNA and affects only part of the product. The feasibility of this method would provide a powerful tool for molecular epidemiology investigations.

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Ultra-Rapid, Simple, Sensitive, and Economical Silica Method for Extraction of Dengue Viral RNA From Clinical Specimens and Mosquitoes by Reverse Transcriptase-Polymerase Chain Reaction

Eliane Chungue, Claudine Roche, Marie-France Lefevre, P. Barbazan, and Suzanne Chanteau

Institut Territorial de Recherches Médicales Louis Malardé (E.C., C.R., M.-F.L., S.C.), and Institut Français pour la Recherche et le Développement Outre-Mer (ORSTOM) (P.B.), Papeete, Tahiti

A rapid, simple and efficient single-tube procedure is described for the isolation of dengue virus RNA from small amount of serum (10 µl) followed by a reverse transcriptase-polymerase chain reaction (RT-PCR). Recovery of RNA is based on the lysing and nuclease-inactivating properties of guanidinium thiocyanate in the presence of silica. The silica RT-PCR can be completed within 5 hours starting from RNA extraction to agarose gel electrophoresis. All of the 63 dengue-3 culture-positive sera were RT-PCR-positive (virus titres: $<10^2$ to $11^{10.69}$). Of 33 culture-negative acute sera from serologically confirmed dengue fever patients collected during dengue-3 epidemic, 4 were RT-PCR-positive. RT-PCR was also positive in 29 of 30 dengue-1 culture-positive sera (virus titres range: $<10^2$ to $10^{8.69}$). Dengue-1 virus was also detected in field-caught *Aedes aegypti* mosquitoes by silica RT-PCR.

KEY WORDS: dengue diagnosis, guanidinium extraction, genomic amplification, RNA isolation, silica RT-PCR

INTRODUCTION

The four serotypes of dengue viruses, designated dengue-1, -2, -3, and -4, are members of the *Flaviviridae*, a family of viruses containing an 11 kilobase single-stranded, positive sense RNA genome. These viruses are transmitted principally by *Aedes aegypti* mosquitoes. They produce a large spectrum of illnesses in humans varying from an acute, self-limiting disease (dengue fever or DF) to very severe syndromes such as ... ical and tropical regions [Halstead ... and Putnak, 1990]. The identification and typing of dengue virus isolated from clinical specimens and field-caught mosquitoes are important for clinical and epidemiological investigations. The classical virus isolation methods followed by serological typing usually take from days to weeks [Gubler et al., 1984]. Although the recent development of the polymerase chain reaction (PCR) has greatly improved the rapidity of the diagnosis of viral diseases [Saiki et al., 1988], the current complexity of nucleic acid extraction methods from biological samples is the major limitation. Several methods for detection of dengue virus in serum by reverse transcriptase-polymerase chain reaction (RT-PCR) have been recently described [Deubel et al., 1990; Henchal et al., 1991; Lanciotti et al., 1992] but the techniques used therein for RNA preparation are still laborious, time-consuming, and difficult to handle while treating many samples.

We now describe a procedure in which the RNA extraction and the RT-PCR are performed in the same microtube. The protocol for the isolation of RNA from a minute quantity of dengue patient serum or mosquitoe-clarified homogenates is ultra-rapid, simple, and highly efficient. The recovery of viral RNA is based on the lysing and nuclease-inactivating properties of the chaotropic agent guanidinium thiocyanate (GuSCN) together with the nucleic acid-binding properties of silica particles [Boom et al., 1990].

MATERIALS AND METHODS

Sera

Acute sera from 93 virologically confirmed dengue fever patients were tested by the silica RT-PCR assay. These samples had previously been shown to contain dengue viruses by isolation in C6/36 *Aedes albopictus* cell cultures [Chungue et al., 1992]. Dengue serotyping was achieved using indirect immunofluorescence assay

with reference virus type-specific monoclonal antibodies [Henchal et al., 1983] kindly provided by Dr. Nick Karabatsos, Centers for Disease Control, Fort Collins. Among them, 63 were identified as dengue-3 and 30 as dengue-1. These samples were collected in French Polynesia during the recent epidemics and stored at −80°C until use. Virus titrations were carried out by using tenfold dilutions of sera inoculated to C6/36 cultivated in 24-well plates. The method of Reed and Muench [1938] was used to calculate the $TCID_{50}$, expressed as the number of 50% infectious doses per ml of serum.

Acute sera from 33 serologically confirmed but virus-negative dengue patients collected during the dengue-3 epidemic [Glaziou et al., 1992] were also tested by the silica RT-PCR assay.

Normal human sera were obtained from 10 healthy French military volunteers within the week of their arrival in Tahiti. The sera were confirmed to be virus-negative by inoculation to C6/36 cells.

Positive controls consisted of sera that had been previously shown to be positive for dengue-3 or dengue-1 viruses both in culture assay and the previously described RT-PCR method [Deubel et al., 1990].

Field-Caught Mosquitoes

Female adult *A. aegypti* and *Aedes polynesiensis* were captured using human bait collection technique [Bonnet and Chapman, 1958] as a part of our dengue surveillance system during the dengue-1 epidemic [Chungue et al., 1992]. Viral assay on C6/36 cells had been carried out routinely on these specimens using clarified homogenates obtained as described previously [Chungue et al., 1992]. Briefly, 24 pools of *A. aegypti* and 7 pools of *A. polynesiensis* mosquitoes were constituted according to species, location, and day of collection. These pools consisted of 2 to 63 mosquitoes (418 in total) and 2 to 14 mosquitoes (54 in total), respectively. After starvation for 24–48 hours, they were ground in chilled 0.05 M, pH 7.6 phosphate-buffered saline (100 µl per mosquito) containing 20% heat-inactivated fetal calf serum and antibiotics. Thereafter, the homogenates were centrifuged at 800 g for 20 minutes at 4°C for clarification. Of 24 pools of *A. aegypti*, 7 had been dengue-1 culture-positive. The 7 pools of *A. polynesiensis* had been negative in culture. RT-PCR assay was carried out on these specimens using 10 µl of the clarified homogenate stored at −80°C until use. Negative controls were made up of 3 pools of ten insectary bred *A. aegypti* mosquitoes.

Silica RT-PCR Procedure

The general flow chart of the procedure is depicted in Figure 1. Ten microliters of serum or mosquito homogenate [illegible] with 90 µl of lysing buffer [illegible]

[illegible] fractionated silica particles (Sigma [illegible] Louis, MO). After 10 minutes incubation at room temperature and 15 sec centrifugation at 12,000 g, the supernatant was removed by suction. Using successive

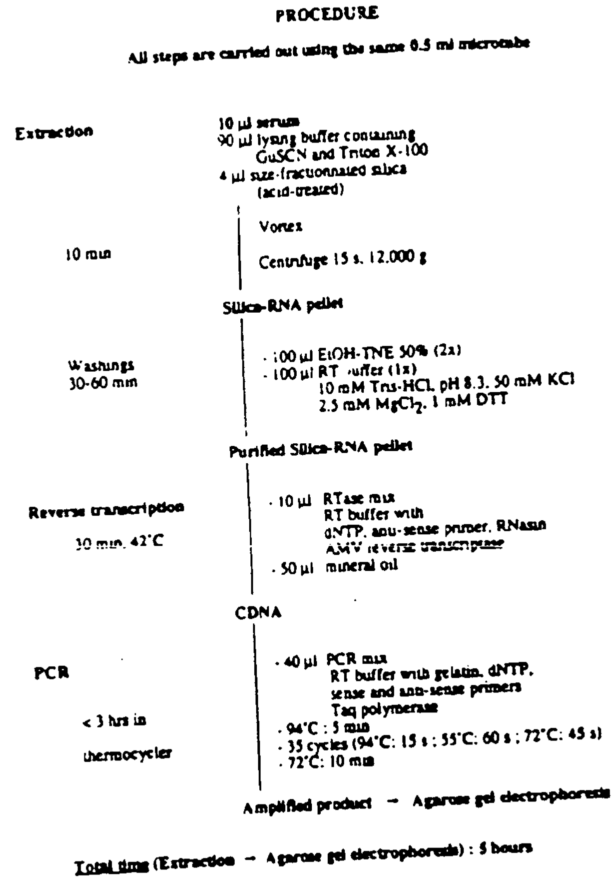

Fig. 1. General flow chart of the RT-PCR procedure.

rapid centrifugations, the silica-RNA pellet was subsequently washed twice with 100 µl of washing buffer (ethanol 50%, 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 50 mM NaCl) and once with 100 µl of RT buffer (10 mM Tris HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol). The reverse transcription was carried out for 30 minutes at 42°C in 10 µl of RT mixture made of RT buffer containing 250 µM each of the four deoxynucleotides triphosphates (dNTP), 250 nM antisense primer, 10 units of RNase inhibitor and 6 units of avian myeloblastosis virus (AMV) reverse transcriptase (Boehringer Mannheim, Germany). The RT mixture was overlaid with 50 µl of mineral oil. Thereafter, 40 µl of PCR mixture containing 10 mM Tris HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, 250 µM each dNTP, 1 µM each primer and 1.25 unit of Taq polymerase (Beckman, Fullerton, CA) were added straight. Type-specific sense and antisense primers [illegible] ded by Dr. Deubel Institut Pasteur, Paris) [illegible]

[illegible] The PCR mixture was heated [illegible] and then cycled 35 times at 94°C (15 sec, 55°C 60 sec and 72°C (45 sec) followed by a final incubation for 10

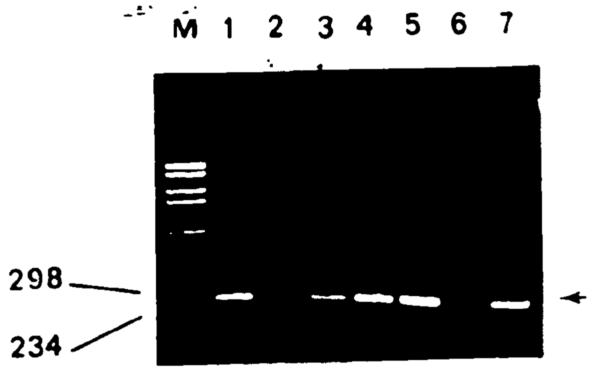

Fig. 2. Ethidium bromide-stained agarose gel for the revelation of RT-PCR dengue-3 virus RNA amplified products. Lanes: M, molecular weight markers in base pairs; 1: positive control; 2: normal human serum; 3–5: serum specimens containing < 10^4, 10^2 and 10^0 $TCID_{50}$/ml, respectively; 6–7: culture-negative sera from serologically confirmed dengue fever patients collected at day 4 and day 1 of illness, respectively. The expected PCR product (257 bp) is indicated with an arrow.

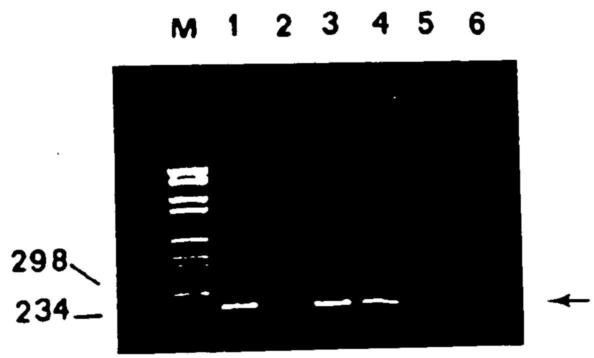

Fig. 3. Ethidium bromide-stained agarose gel for the revelation of RT-PCR dengue-1 virus amplified products. Lanes: M, molecular weight markers in base pairs; 1: positive control; 2–4: culture-positive pools of field-caught *Aedes aegypti* mosquitoes; 5: culture-negative pool of *A. aegypti*; 6: negative control. The expected PCR product (237 bp) is indicated with an arrow.

min at 72°C. The amplified products at predicted size (257 bp and 237 bp for dengue-3 and dengue-1, respectively) were revealed by staining with ethidium bromide after agarose gel electrophoresis of 5 μl of sample aliquots.

RESULTS

Typical results are shown in Figures 2 and 3. By using this fast extraction method (30–60 minutes) fol- [illegible] negative for dengue-3 and dengue-1. [illegible] dengue-3 culture-positive sera tested were RT-PCR-positive (100% sensitivity, 100% agreement rate). The detection limit was very low as virus titres of some sera on C6/36 culture were inferior to 10^2 of 50% infective doses per ml ($TCID_{50}$/ml). Moreover, RT-PCR was also positive in 4 of 33 culture-negative acute sera from serologically confirmed dengue fever patients. Of 30 dengue-1 culture-positive acute sera, 29 were RT-PCR-positive (96% sensitivity, 97% agreement rate). As for dengue-3, the detection limit for dengue-1 RT-PCR was at $TCID_{50}$/ml inferior to 10^2. Sensitivity and agreement rate of the results compared to the culture assay were also very high with field-caught mosquitoes. All the culture-positive *A. aegypti* mosquito samples were RT-PCR-positive and the culture-negative samples were negative (100% sensitivity, 100% agreement rate). All the 7 culture-negative *A. polynesiensis* specimens were RT-PCR-negative.

DISCUSSION

We describe a sensitive, rapid, simple, and economical silica RT-PCR procedure for the diagnosis of dengue infections in human sera and in mosquitoes. The sensitivity of the silica RT-PCR method is greater or equal to methods recently published [Henchal et al., 1991; Lanciotti et al., 1992]. The main feature of our procedure is the simplicity of the viral RNA extraction that allows efficient RT-PCR. It has the advantage of eliminating the need of proteolysis with proteinase-K and extractions with phenol/chloroform and ethanol precipitation. Serum samples with virus titres lower than 10^2 $TCID_{50}$/ml were positive and 12% of the culture-negative acute serum samples from serologically confirmed dengue patients were also found positive by using this method. The agreement between RT-PCR and culture was excellent. However, there was one false negative out of 30 dengue-1 sera (4%); it concerned a very low titred serum ($TCID_{50}$/ml < 10^2). Others have reported similar levels of false negative results related to either low positivity on culture assays [Lanciotti et al., 1992] or presence of inhibitory components especially in long storage history specimens.

PCR or RT-PCR assays on insects usually pose the problem of inhibitory components which compromise the enzyme activities and lead to false negative results. Additional purification steps such as dialysis or other sophisticated techniques are often required to purify the RNA or DNA extracts [Dissanayake et al., 1991; Meredith et al., 1991]. For silica RT-PCR on dengue-infected mosquitoes, there are no additional purification steps of the RNA extract prior to RT-PCR, such as RNA capture described elsewhere [Lanciotti et al., 1992]. The use of silica combined with the guanidinium thiocyanate could circumvent loss of sensitivity possibly due to unidentified inhibitory components that might be present in biological specimens. The sensitiv- [illegible] silica RT-PCR for the detection of dengue [illegible] detecting dengue [illegible] maintained [illegible] aegypti mosquitoes [illegible] viremic patient (data not shown).

Excluding the thermocycler, special laboratory equipment is not needed and sources of contamination

due to multiple transfers of the samples are dramatically reduced. Therefore, this method is foreseen to be particularly useful in endemic countries for urgent diagnosis of dengue and for surveillance of vectors. Moreover, the detection of other RNA viruses, especially other flaviviruses, may be envisaged using this technique.

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